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(54) Title: REGULATION OF HAIR FOLLICLE MORPHOGENESIS BASED ON BETA-CATENIN

(57) Abstract

The present invention provides a method for inducing hair growth by providing beta-catenin activity to a skin cell. This may be achieved by providing a beta-catenin polypeptide, providing a beta-catenin agonist, providing a polynucleotide encoding a beta-catenin polypeptide, enhancing the de novo synthesis of beta-catenin, increasing the stability or decreasing the degradation of beta-catenin polypeptides.

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DESCRIPTION

REGULATION OF HAIR FOLLICLE MORPHOGENESIS BASED ON BETA-CATENIN

BACKGROUND OF THE INVENTION

This invention claims the benefit of U.S. Provisional Patent Application serial number 60/109,284, filed November 20, 1998, the entire text of each of which is specifically incorporated by reference herein without disclaimer. The government owns rights in the present invention pursuant to grant numbers AR31737 and 5P50-DE11921 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of the hair growth and morphogenesis. More particularly, it concerns the ability of β-catenin to induce the post natal *de novo* formation of hair follicles replete with sebaceous glands and dermal papilla.

2. Description of Related Art

A. Hair Morphogenesis

Hair morphogenesis is dependent upon a series of mesenchymal-epithelial interactions in embryonic skin (Hardy, 1992). The first signal is mesenchymal, instructing ectoderm to form a hair germ. An ectodermal signal then alters associated mesenchyme to condense and form a dermal papilla, developed by the ectoderm. Finally, a second dermal signal initiates proliferation and differentiation within the developing epithelial component to form the follicle and its sebaceous gland appendages.

Once established during embryogenesis, the dermal papilla and upper portion of the follicle (including the sebaceous gland) are permanent in postnatal life, but the remainder of the follicle undergoes cycles of growth (anagen), regression (catagen), and rest (telogen) (FIG. 1; Hardy, 1992). Each time a follicle regresses, the dermal

papilla contracts upward, coming into transient contact with the permanent epithelial base, called the bulge. This mesenchymal-epithelial interaction stimulates stem cells near or at the bulge to proliferate, reform the lower epithelial portion of the follicle and produce the hair (Cotsarelis *et al.*, 1990; Wilson *et al.*, 1994; Kobayashi *et al.*, 1993). In these cycles, the embryonic dermal signal (to make an appendage), and ectodermal signal (to make a dermal papilla) are no longer needed, but the dermal papilla cells must periodically transmit a signal to the bulge cells to initiate a new hair follicle (Hardy, 1992).

The inductive signals exchanged among epithelial components within the follicle and between follicular epithelia and mesenchyme are largely unknown; however, recent studies suggest that lymphoid enhancer factor *Lef-1* might be involved. *Lef-1* mRNAs are first expressed in E12.5 ectodermal placodes, and as the developing follicle assembles a dermal papilla, epithelial *Lef-1* is maintained at the growing tip of the hair germ (Zhou *et al.*, 1995). During hair germ formation, it is also induced in the mesenchyme at the base of the follicle (Zhou *et al.*, 1995; Kratchowil *et al.*, 1996). That *Lef-1* plays some role in follicle development is demonstrated convincingly by *Lef-1* null mice, which display a reduction and shortening of hair coat follicles and lack whiskers (van de Genderen *et al.*, 1994). Mesenchymal *Lef-1* may be important in whisker formation, as the process is blocked in embryonic organ culture even if wild-type ectoderm is combined with *Lef-1* null mesenchyme (Kratchowil *et al.*, 1996). Epithelial *Lef-1* may be important in some aspect of the hair coat, since K14-*Lef-1* transgenic mice display perturbations in the orientation of whisker and body hairs (Zhou *et al.*, 1995).

One difficulty that precludes evaluation of the relative importance of epithelial and mesenchymally expressed *Lef-1* in follicle formation stems from the fact that whiskers are specialized follicles that share some, but not other, regulatory mechanisms with body hair follicles. The *hairless* mutant mouse, for instance, has wild-type whiskers, but severe defects in hair coat (Cachon-Gonzalez *et al.*, 1994). Interpretations of the knockout studies may be further hampered if other members of the *Lef-1/Tcf* family are expressed in follicles. Superimposed on these complex issues

are two more general questions, presently unanswered: Which step(s) in the pathways of hair follicle morphogenesis, cycling and/or growth is controlled by *Lef-1/Tcfs*? How does *Lef-1/Tcf* function to regulate follicle formation?

While the downstream targets of mammalian *Lef-1/Tcfs* have yet to be identified in non-lymphoid cells, some mechanistic insights into *Lef-1/Tcf* regulation have surfaced through the recent discovery that these factors can partner with β-catenin and function in the *Wnt/wingless* pathway (for review, see Clevers and van de Wetering, 1997; Gumbiner, 1997; Willert and Nusse, 1998). Activation by *Wnt/wingless* leads to inhibition of the GSK3 kinase that phosphorylates β-catenin's N-terminus and typically targets its cytoplasmic pool for ubiquitin-mediated degradation (Munemitsu *et al.*, 1996; Aberle *et al.*, 1997). Upon accumulation, β-catenin then interacts with members of the Lef1/Tcf family of DNA binding proteins to generate a functional transcription factor complex (Behrens *et al.*, 1996; van de Wetering *et al.*, 1997; for review, see Clevers and van de Wetering, 1997; Willert and Nusse, 1998).

A number of investigators have circumvented the normal requirement for *wnt/wingless* signaling by engineering an N-terminally truncated form of β-catenin, which *in vivo* is stabilized in a constitutive fashion (Funuyama *et al.*, 1995; Munemitsu *et al.*, 1996; Wylie *et al.*, 1996; van de Wetering *et al.*, 1997; Fagotto *et al.*, 1998; Wong *et al.*, 1998). This form can still interact with *Lef-1/Tcf* family members and function as a transcription co-factor (Molenaar *et al.*, 1996), which is likely to account for its effects on proliferation in intestinal epithelia (Wong *et al.*, 1998) and on axis duplication in *Xenopus* development (Funuyama *et al.*, 1995; Wylie *et al.*, 1996; Fagotto *et al.*, 1998). Whether the *wnt/wingless* pathway functions in hair follicle morphogenesis and/or hair cycling has not yet been explored. Both *Lef-1/Tcf* members and β-catenin can be activated independently of a *Wnt/wingless* signal (Cadigan and Nusse *et al.*, 1997; Willert and Nusse, 1998; Novak *et al.*, 1998).

β-catenin can also act independently of Tcf/Lef, where it plays an essential role in cell-cell junction formation (for reviews, see Klymkowsky and Parr, 1995; Barth *et al.*, 1997). In many epithelia, β-catenin binds to cell surface E- and P-cadherins and also to α-catenin, forming a bridge to the actin cytoskeleton (Pasdar and Nelson, 1988; Adams *et al.*, 1996). Microinjection of antibodies against P- and E-cadherins interfere with hair follicle morphogenesis *in vitro* (Hirai *et al.*, 1989), reflective of the extensive remodeling of epithelial cell junctions that occurs during this process.

B. Alopecia and Hair Growth

Current knowledge does not permit full understanding of all types of alopecia. These disorders have been categorized into two classes, cicatrical (scarring) and noncicatrical. Cicatrical alopecias result from the malformation or destruction of follicles so that they cannot produce hair caused by developmental defects, *e.g.*, aplasia cutis congentia, infection, neoplasms and chemical and physical agents, *e.g.*, burns and mechanical trauma. Noncicatrical alopecias result from either the unscheduled entry of many follicles into the telogen stage of the hair cycle or the transformation of terminal to vellus follicles resulting in short, fine, unpigmented hairs. The class of noncicatrical alopecias include male pattern baldness (androgenetic alopecia) and alopecia areata. Currently, minoxidil can help prevent or retard hair loss in male pattern baldness. It must be used constantly and tends to be effective only on individuals who have early balding of the vertex that is less than 10 cm in diameter. Treatments for alopecia areata include high-potency corticosteriods and contact allergens, primary irritants, and the photosensitizing drug psoralen. All these therapies have limited efficacy and potential side effects. There is a current need for new therapies effective for a range of alopecia disorders. In addition to induction of human hair growth, delivery of β-catenin activity may be used to increase the density of hair growth in sheep and other livestock. There is a current need for methodologies that improve wool production from livestock.

Methods for the removal of hair are currently limited to physical depilation. With most physical depilation methods there is a regrowth of the removed hair. There

is a current need for non-physical methods to prevent hair growth that do not initiate the subsequent regrowth of hair.

SUMMARY OF THE INVENTION

The present invention provides a method for inducing hair growth by providing β-catenin activity to a skin cell. This may be achieved by, but not limited to, providing a β-catenin polypeptide, providing a β-catenin agonist, providing a polynucleotide encoding a β-catenin polypeptide, enhancing the *de novo* synthesis of β-catenin, increasing the stability of β-catenin polypeptides or otherwise decrease the degradation of β-catenin polypeptides. A polynucleotide encoding a β-catenin polypeptide may be provided by gene therapy and, in the case of sheep and other livestock, by production of a transgenic animal. As used herein, the term "β-catenin" and β-catenin polypeptide is intended to refer to a polypeptide based on the native sequence of β-catenin. This includes wild-type β-catenin and analogs and derivatives of wild-type β-catenin which retain the activity to induce hair growth. In one embodiment of the invention the β-catenin polypeptide is ΔN87βcat.

Thus, in certain aspects, including but not limited to, a β-catenin polypeptide, a β-catenin agonist, a polynucleotide encoding a β-catenin polypeptide, enhancing the *de novo* synthesis of β-catenin, increasing the stability of β-catenin polypeptides or otherwise decrease the degradation of β-catenin polypeptides, for use as a medicament is contemplated.

In some aspects, including but not limited to, an inhibitor of the Wnt or Shh signaling pathway, such as an inhibitor of mitogen-activated protein kinase, recombinant FrzB, constitutively active forms of GSK, dominant negative β-catenin polypeptides and dominant negative *Lef-1* polypeptides, for use as a medicament is contemplated.

In other aspects, including but not limited to, use of a β-catenin polypeptide, a β-catenin agonist, a polynucleotide encoding a β-catenin polypeptide, enhancing the

de novo synthesis of β-catenin, increasing the stability of β-catenin polypeptides or otherwise decrease the degradation of β-catenin polypeptides, for the manufacture of a medicament for the treatment of disease, including but not limited to a condition such as reduced hair growth or morphogenesis (*i.e.*, alopecia).

In additional aspects, including but not limited to, use of an inhibitor of the Wnt or Shh signaling pathway, such as an inhibitor of mitogen-activated protein kinase, recombinant FrzB, constitutively active forms of GSK, dominant negative β-catenin polypeptides and dominant negative *Lef-1* polypeptides, for the manufacture of a medicament for the treatment of disease, including but not limited to a condition such as excess or undesired hair growth or morphogenesis and cancer, particularly hair tumors.

The β-catenin activity may be provided in a pharmaceutically acceptable therapeutic composition. Administration of therapeutic compositions according to the present invention may be *via* any route so long as the target tissue is available *via* that route. In a preferred embodiment, the therapeutic composition may be applied topically. A topical composition may be formulated as, but not limited to, a cream, lotion, emulsion or be aqueous or non-aqueous based. In some embodiments of the invention the topical formulation contains liposomes. In some embodiments of the invention the liposomes may contain a ligand. The ligand may be a galactose-terminal asialganglioisde. The invention also provides that liposomes may contain proteins including, but not limited to, HJV and nuclear nonhistone chromosomal proteins. In other embodiments of the invention, the topical formulation contains cationic lipid-DNA lipoplexes.

The invention also provides for the use of methods to maximize the delivery of β-catenin activity by topical application. Both chemical and biophysical methods may be used to enhance the bioavailability of topically applied therapeutic agents. In one embodiment the superficial layers of the skin are removed by tape stripping. In other embodiments the invention provides for the use of intophoresis or related methods using electrical fields to enhance percutaneous absorption.

In other embodiments the β-catenin activity may be delivered by parenteral administration. Parenteral administration may be achieved by, but not limited to, intradermal, subcutaneous, or intravenous injection. The invention also provides that the β-catenin activity may be provided by ballistic transfer. The invention further provides for *ex vivo* delivery of β-catenin activity. In one embodiment of the invention a patient's skin cells are cultured, transduced *in vitro*, then returned to the patient. The skin cells can be returned to the patient by intradermal injection or by autlogous skin graft after culturing skin cells as epithelial sheet.

The β-catenin activity may be provided to the skin cell by means of a polynucleotide. The polynucleotide may encode a β-catenin polypeptide or a β-catenin polypeptide analog or derivative. In one embodiment of the invention, the polynucleotide is operably linked to a promoter. The invention provides that the promoter may be skin specific and may be selected from the group consisting of keratin promoters, involucrin promoters, filagrin promoters and loricrin promoters. The invention further provides that keratin promoters may be selected from the group consisting of K3, K5, K10, K14 and K16. In preferred embodiments of the invention, the keratin promoter is K5 or K14. In other embodiments of the invention, the promoter may be selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, tetracyline regulatable and ecdysone regulatable. The promoter may also be inducible.

A polynucleotide may be contained in a vector. The vector may be a viral vector. The invention provides that a viral vector may be selected from the group consisting of adeonvirus, retrovirus, adeno-associated virus, vaccinia virus and polyomavirus. The vector may be a non-viral vector. Non-viral vectors include, but are not limited to, DNA loaded liposomes, cationic lipid-DNA lipoplexes and cationic polymer-DNA complexes. The invention also provides for the delivery of plasmids or naked DNA. In various embodiments of the invention, non-viral *in vivio* and/or *ex vivo* transfer of expression constructs may be achieved by, but not limited to, calcium

phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, cell sonication, ballistic transfer, receptor medicated transfection or use of liposomes, lipoplexes or polymer complexes.

The β-catenin activity may be provided by stabilising endogenous β-catenin. Endogenous β-catenin may be stabilized by inhibition of glycogen synthase kinase 3 by such factors as, but not limited to, the products of Wnt genes, insulin, epidermal growth factor and integrin linked kninase.

The invention also provides a method where β-catenin activity is provided in conjunction with a factor that triggers the induction of hair growth by β-catenin. This factor may be provided by a method that induces existing hair follicles to enter a synchronous hair cycle. Hair follicles may be induced to synchronously enter the anagen stage of the hair cycle by depilation or by administration of chemical agents such as cyclosporin or FK506. The invention also provides that β-catenin activity may be provided with an agent that provides *Lef-1* or TCF-3activity to the skin cell.

The invention provides a method for screening for hair growth disorders and/or the propensity for developing alopecia comprising evaluation of β-catenin expression in a skin cell. The expression of β-catenin polypeptides can be evaluated by immunodetection methods such as enzyme linked immunosorbent assays and immunohistochemistry. Alternatively, β-catenin mRNA may be evaluated by methods such as RT-PCR™. The invention also provides methods for screening for hair growth disorders and the propensity for developing alopecia comprising evaluation of the responsiveness of a skin cell to β-catenin activity. This screening may consist of providing the skin cell with β-catenin activity and evaluating the localization of β-catenin or *Lef-1* polypeptides in the nucleus of the skin cell by immunohistochemical techniques or by evaluating the activation of the c-*MYC* gene, a newly identifed target gene of the β-catenin pathway.

The invention provides for a screening method for compounds that may deliver β-catenin activity. Induction by a compound of the translocation of *Lef*-1 to the nucleus of a cell is indicative of the delivery of β-catenin activity. Translocation of *Lef-1* to the nucleus of a cell can be established by immunohistochemical techniques and can be adapted for high through-put screening of candidate compounds. This screening may be facilitated by the use of the reporter construct TOPFLASH. This reporter contains the luciferase gene under the control of an enhancer containing *Lef-1*/Tcf elements. Thus, however induced, nuclear β-catenin together with *Lef-1* will activate the reporter which is readily monitored by a luminometer. This assay is readily adaptable for large scale screening using, for example, microtiter plate assay methodologies. The same enhancer linked to the GFP protein may also be used for screening.

The invention provides a method for inducing *de novo* hair morphogensis comprising providing β-catenin activity to a skin cell. The invention also provides methods for the treatment of alopecia, stimulating hair growth, and preventing hair loss comprising providing β-catenin activity to a skin cell.

The invention provides for a method of inhibiting hair growth. Any method that leads to an inhibition of the Wnt signaling pathway in hair should lead to an inhibition of hair growth. Such inhibitors may include, but not be limited to, inhibitors of mitogen-activated protein kinase, recombinant FrzB, constitutively active forms of GSK, dominant negative β-catenin polypeptides and dominant negative *Lef-1* polypeptides. The above described high through-put screening methods could be used for identification of inhibitors of hair growth, which would decrease reporter activation.

The transgenic ΔN87βcat phenotype mouse exemplified in the present invention provides a model for screening compounds and therapies for human hair tumors, including trichofolliculoma and pilomatricoma. The benefit of this model is

that ΔN87βcat mice exhibited numerous trichofolliculomas and pilomatricomas were numerous and occurred in a predictable fashion.

The invention additionally provides a method of sensitizing at least one cell to at least one nucleic acid damaging agent, comprising administering to the patient(s) at least one effective amount of at least one dominant/negative APE/Ref-1 polypeptide or nucleic acid that encodes the dominant/negative APE/Ref-1 polypeptide(s).

In keeping with the use of longstanding patent terminology, the terms "a" or "an", when used with the term "comprising", "comprises", "includes" or "including", may mean one or more than one herein the specification and claims.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Hair structure. The sebaceous gland, upper outer root sheath (including the bulge) and mesenchymally derived dermal papilla are established once, during embryonic follicle morphogenesis. The lower portion cycles postnatally by a process involving transient stimulation of bulge stem cells by dermal papilla (Cotsarelis *et al.*, 1990; Wilson *et al.*, 1994). Matrix cells are transit amplifying cells

which differentiate upward in concentric rings of cells, giving rise to precortex, cortex and medulla (hair shaft), and surrounded by IRS and ORS.

FIG. 2. pTOPFLASH luciferase reporter assays. Mouse keratinocytes were transfected with pTOPFLASH, containing four consensus Tcf/Lef binding motifs, a minimal Fos promoter and the luciferase reporter gene (van de Wetering *et al.*, 1997; Korinek *et al.*, 1998a). Transfections were done with or without K14-ΔN87βcat and CMV-hLef-1. Forty-eight hour posttransfection, cells were lysed and protein extracts were assayed for luciferase. Studies were repeated 3 times, each time with a second reporter construct, CMV- lacZ, to correct for plasmid transfection efficiency. Luciferase activity values (in light units) represent an average of the three studies, with variations shown by error bars.

FIG. 3 Stick diagram of K14-ΔN87βcat. Abbreviations: K14, 2100 bp of K14 promoter sequence (Vassar *et al.*, 1989); arm; armadillo repeats; B, *Bam*HI; X, *Xho*I; Bg, *Bgl*II; int, β-globin intron; pA, polyadenylation signal.

FIG. 4. Activation of the LEF/TCF-dependent TOPGAL transgene in keratinocytes and in transgenic mice that also express a stabilized form of þ-catenin. (A) TOPGAL construct. The promoter contains three consensus LEF-1/TCF-binding motifs (L) and a minimal *c-fos* promoter to drive transcription of the *lacZ* gene encoding þ-galactosidase. (B) pTOPGAL reporter assays. The mouse keratinocyte line, UG1, was transfected with pTOPGAL, pCMV-luciferase (as an internal control gene) and equimolar amounts of plasmids pK14-• • • • þcat (K14-þ• þcat), pK14-Lef1 or empty expression vector, as indicated (see Gat *et al.*, 1998 for method). 48 hours later, cells were lysed and protein extracts were assayed for þ-galactosidase activity (test) and luciferase (to correct for transfection efficiency). Normalized activities represent an average of three experiments, with variations shown by error bars. FOPGAL had no activity (not shown).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The inventors have used a tissue-specific K14 promoter to drive the expression of an N-terminally truncated human β-catenin mutant, ΔN87βcat, in skin. The results indicate that β-catenin is a key factor in controlling hair follicle morphogensis. The expression of ΔN87βcat in the basal layer of the epidermis and follicle outer root sheath reprograms these cells to induce hair follicle morphogenesis. The inventors have shown that the process of hair follicle morphogenesis occurs, including the development of dermal papilla and sebaceous glands normally established only in embryogenesis, and hair shaft production typical of both initial and cycling follicles. As *de novo* induction of hair follicles will result in the growth of new hairs, the invention will be useful therapy for hair growth and alopecia disorders in humans and the induction of hair growth in sheep and other livestock.

A. Wingless/Wnt pathway and β-Catenin

The wingless cascade, first identified in Drosophila, has been implicated in a multitude of developmental processes. There are at least 11 known Wnt genes in vertebrates which are homologs of the Drosophila Wingless genes (Parr & McMahon, 1994). The Wnt proteins are secreted polypeptides, about 350-380 amino acids long and have a signal sequence and a recognition site for a signal peptidase. They are known to tightly associate with the extracellular matrix thus they may function mostly in short distance signaling. Mutations in Drosophila which have similar phenotypes to those of wingless and biochemical studies have identified the components of an entire pathway transducing the wingless signals. It is thought that wg/wnt bind and transduce their message to a family of seven transmembrane domain (serpentine) receptors called Frizzled (fz).

The message is then transmitted in an unknown way to a cytoplasmic protein called Disheveled (dsh) of which there also several homologs in vertebrates. Disheveled is hyperphosphorylated upon activation and probably translocates to the membrane, a process which is thought to result in phosphorylation and inactivation of a usually constitutively active kinase - Glycogen Synthase Kinase 3 (GSK3 or ZW3 in

Drosophila). GSK3 is an ubiquitous serine/threonine kinase involved in multiple diverse functions such as basic metabolism, growth control and cell fate decisions (Woodgett *et al.*, 1993). GSK is known to have many substrates, including the transcription factors Jun and Fos as well as Myc and Myb and the microtubule associated protein Tau. Typically, phosphorylation by GSK causes inactivation of its substrate. However, GSK's major target in the Wnt pathway is thought to be β-catenin, the mammalian homolog of the Drosophila Armadillo protein.

Beta-catenin has two known functions: (1) it localizes to intercellular adherens junctions, where it binds to cadherins and participates in stabilizing them by linking them to the actin cytoskeleton (through α-catenin); and (2) when cytoplasmic pools of β-catenin accumulate, it can associate with the Lef-1/Tcf family of DNA binding proteins, and translocate to the nucleus, and transactivate the expression of genes that are regulated by Lef-1/Tcf sequence motifs. When the Wnt pathway is inactive, GSK causes the phosphorylation of β-catenin, which in turn leads to its ubiquitination and proteolysis, thereby preventing accumulation of free cytoplasmic pools of the protein. Upon activation of the Wnt pathway, GSK is inhibited, leading to stabilization of cytoplasmic β-catenin and its ability to associate with Lef-1/Tcf proteins to become a transcription factor. This in turn causes Wnt target genes to be transcribed and a Wnt developmental program is executed.

B. β-Catenin and Hair Morphogenesis

Using a ΔN87βcat expression vector, the inventors demonstrated the functionality of ΔN87βcat in keratinocytes. β-Catenin accumulated in the cytoplasm of transfected keratinocytes. Upon elevating the levels of *Lef-1* in these keratinocytes, most of the β-catenin concentrated in the nuclei. Thus ΔN87βcat can localize to sites of epidermal cell-cell adhesion, and if sufficient *Tcf/Lef* is present, it can translocate to the nucleus. This is a key component of the wingless signaling pathway (Molenaar *et al.*, 1996; Larabell *et al.*, 1997; Fagotto *et al.*, 1998).

In K14-ΔN87βcat transgenic mice, ΔN87βcat accumulated in the skin. Epithelial invaginations began to appear at the onset of the second hair cycle in the transgenic mice. As the invaginations grew they resembled embryonic hair germs. Biochemical and ultrastructural studies provide unequivocal evidence the epidermal invaginations lead to bona fide hair follicles replete with markers of hair shaft formation.

Even though normal postnatal follicles cycle through resting and growth phases, they always retain and reutilize the dermal papilla, sebaceous glands and upper ORS that were established early in development (Hardy, 1992). In contrast, many of the ΔN87βcat-induced epithelial invaginations assembled and engulfed mesenchymal condensates, each creating their own dermal papilla and sebaceous gland. These *de novo* hair bulbs subsequently differentiated to produce an outer and inner root sheath and hair shaft. The inventors' data suggest that transient activation of β-catenin may be the long-sought "epidermal message" which has been predicted to receive a "dermal message" and respond by assembling a dermal papilla and forming an epithelial bulb around it (Sengel, 1990; Hardy, 1992).

Another factor clearly important in eliciting a Wnt signal in hair follicle induction is Lef-1/Tcf expression. In the presence of Lef-1, β-catenin can collaborate to transactivate a reporter gene containing Lef-1/Tcf sequence motifs 5′ from a minimal enhancer inkeratinoctyes. Overexpression of Lef-1 in transgenic mice can elicit epithelial invaginations (Zhou *et al.*, 1995). These results indicate that β-catenin and *Lef*-1 work together to elicit hair follicle formation in postnatal skin.

The lack of ΔN87βcat function in embryogenesis can be attributed to either or the combination two factors: 1) the K14 promoter, while active at E9.5, is not dramatically elevated until E13.5-E14.5, after the major onset of follicle morphogenesis (Byrne *et al.*, 1994; Wang *et al.*, 1997), and 2) to elicit a response, ΔN87βcat seemed to require a transiently induced factor, which in postnatal skin appeared to be released at the start of the hair cycle. That major morphological

changes in transgenic mice were not observed until initiation of the hair cycle, indicates that β-catenin stabilization is not required at other times during follicle morphogenesis. Thus only the transient expression of β-catenin activity, when such expression occurs in conjunction with other appropriate factors, is required to induce post-natal hair morphogenesis.

In one embodiment of the present invention, a stable β-catenin is delivered to epidermal cells by using an epidermal keratin promoter/enhancer to drive expression of an N-terminally truncated form of β-catenin, lacking the phosphorylation/ubiquination sites. Alternatively, endogenous β-catenin expressed by epidermal cells may be stabilized by inhibition of GSK3. Wnts can act on a cell with the appropriate receptor and lead to inactivation of GSK3 and stabilization of β-catenin. GSK3 is also inhibited by insulin and epidermal growth factor signaling *via* its phosphorylation primarily by the MAPK (mitogen activated protein kinase) cascade (Seedorf *et al.*, 1995). Inhibition of GSK3 is also implicated in the mechanism by which integrin linked kinase increases cytoplasmic levels of β-catenin (Novak *et al.*, 1998). This kinase is activated by cell-extracellular matrix interactions and is a proto-oncogene whose overexpression causes anchorage independent growth of cells

Factors that stabilize β-catenin include the products of the Wnt genes. Wnt10b expression has been reported in skin (Wang *et al.*, 1996) and during development of the hair follicle (St-Jacques, 1998). Although this particular member seems to be the prime candidate to be involved in hair formation other Wnt genes such as Wnt7a, Wnt3a may also be involved in one the multiple stages and may activate β-catenin stabilization in the skin.

In many cell types, β-catenin degradation is enhanced by association with negative regulatory proteins, including the tumor suppressor gene product APC and recently described proteins axin and conductin. Inactivating mutations of APC cause the accumulation of β-catenin in a Wnt induced like fashion (Polakis, 1997). Thus,

β-catenin stabilization can be enhanced in an epidermal cell by inhibition of these and other negative regulators.

The delivery of β-catenin activity can be used to induce hair growth in sheep and other livestock in addition to humans. A number of different transgenic sheep have been produced (*e.g.*, Schnike *et al.*, 1997; Clements *et al.*, 1994. 1996; Wall *et al.*, 1996). Genes have already been directed to the hair follicle in transgenic sheep (Damak *et al.*, 1996a, 1996b; Su *et al.*, 1998). In one embodiment of the present invention, β-catenin activity is delivered to the epidermal cells of livestock by production of a transgenic animal with a transgene expressing a β-catenin polypeptide.

In converse to stimulating the induction of hair growth by delivery of β-catenin activity, the reverse approach, *i.e.*, use of an inhibitor or suppressor of β-catenin activity, may be used to inhibit hair growth. In a number of cosmetic settings, hair growth inhibition is desirable to suppress hair growth in certain body sites. Any method that leads to an inhibition of the Wnt pathway/growth factor pathway should lead to an inhibition of hair growth. The pathway could be inhibited by using known inhibitors, such as are available for mitogen-activated protein kinase (Carter *et al.*, 1998; Potchinsky *et al.*, 1998; Nair *et al.*, 1997). In addition, gene therapy of the Wnt cascade may be used, such as application of recombinant FrzB, a Frizzled receptor inhibitor (Lin *et al*, 1997); use of a constitutive active form of GSK, which could be elicited by phosphorylation; a dominant negative β-catenin, which does not contain the trans-activating C-terminal domain or a dominant negative Lef-1 which does not contain the N-terminal domain required for β-catenin binding. Inhibition of β-catenin activity will stop progression of hair through the next hair cycle and thereby reduce hair density.

C. A Second Factor Augments β-Catenin's Role In Hair Morphogenesis

In all situations, the appearance of invaginations in the ΔN87βcat phenotype, coincided with the start of the first postnatal hair cycle. Both temporally and

regionally, many keratinoctyes that were highly K14 promoter-active did not undergo this change, indicating that some additional inductive factor(s) associated with the synchronized hair cycle was necessary to trigger the ΔN87βcat response. Since the downstream consequences of ΔN87βcat activation in the epidermis and ORS are proliferation and invagination rather than increased cell-cell adhesion, ΔN87βcat probably exerts its effect in conjunction with a *Lef-1/Tcf* factor.

Since the natural levels of *Lef-1/Tcf* are low in keratinoctyes, the molecular trigger might stimulate the production of one of β-catenin's transcriptional cofactors. The inventors' finding that *Lef-1* is upregulated in these early invaginations is consistent with this notion.

The transgenic ΔN87βcat phenotype mouse model can be used as a screen to identify the second factor. Candidate factors can be delivered to the epidermis prior to the initiation of the first postnatal hair cycle. As ΔN87βcat is already being expressed, delivery of the second agent or a compound with equivalent activity will initiate hair morphogenesis prior to that initiated by the first postnatal hair cycle.

The second factor may normally be present in adolescent and adult human and adult skin. However, alopecia covers a diverse range of pathologies and it is possible that the second factor may be present in some cases and not others. Where the second factor is absent, such activity can directly delivered to the skin once the identity of the second agent(s) is(are) established. Alternatively, endogenous second agent activity may be induced by initiation of a synchronous hair cycle. Human hair follicles cycle asynchronously, but follicles can be induced to synchronously move into anagen by depilation or treatment with cyclosporin or FK506 (Paus *et al.*, 1998; Jiang *et al.*, 1995). In one embodiment of the present invention, β-catenin activity is delivered to skin cells in conjunction with a physical or chemical treatment that induces existing hair follicles to synchronously enter the anagen stage of the hair cycle. In another embodiment of the present invention, β-catenin is given in concert with an agent that upregulates or delivers *Lef-1* activity to a skin cell.

D. Sonic Hedgehog Pathway and Hair Growth

Misregulation of sonic hedgehog, which acts through its receptor Patched, has recently been implicated in basal cell carcinomas in humans. The present invention show that activation of sonic hedgehog and patched occurs shortly after hair follicle morphogenesis is induced by β-catenin stabilization/Lef-1 activation. Moreover, in the hair follicle tumors that arise in the mice, patched and sonic hedgehog expression are dramatically elevated. Taken together, these studies suggest that factors that may enhance sonic hedgehog pathway signaling might also stimulate hair follicle growth. In this case, however, the studies suggest that Shh action is downstream from β-catenin stabilization, and therefore, the studies indicate that Shh pathway stimulation would be most useful for enhancing hair growth, rather than for hair follicle formation per se. Nevertheless, in cases where hair growth stimulation is sufficient (*e.g.*, as in minoxidil applications) and where hair follicle formation (*e.g.*, as in hair transplants) is not required, Shh pathway stimulation would be expected to provide some benefit for hair growth, based upon the studies.

Shh pathway inhibitors can be expected to inhibit hair growth. Thus Shh pathway inhibitors, such as cyclopin, may be useful for the treatment or prevention of hair tumors.

E. β-Catenin and Tumorigenesis

Because Wnt pathway activation leads to stimulation of cell growth, uncontrolled or too much stimulation via the Wnt pathway has been suspected of causing cancer. In 85% of colorectal cancers, the tumor suppressor adenomatous polyposis coli ("APC") gene is lost or inactivated. Inactivation of the APC gene leads to β-catenin accumulation in the nucleus and presumably stimulation of tumor cell growth. Wnt signaling has now been linked to activation of the c-*MYC* oncogene (Pennisi, 1998).

Continuous overexpression of ΔN87βcat in the transgenic mouse model is associated with the formation of tumors. Epithelioid cysts were noted in the skin of

3-4 month old animals. These epithelioid cysts bore a striking resemblance to human trichofolliculomas, well-differentiated hair tumors each typified by an epithelioid cyst containing densely packed and misangled hair follicles, and small hairs that emanate from cysts at odd angles relative to the skin surface (Fitzpatrick *et al.*, 1993). This striking resemblance leads to the postulate that an alteration of a gene involved in β-catenin regulation may be causative for trichofolliculoma tumors in humans. As ΔN87βcat mice aged, they developed visible, large tumors (>1 cm dia.), which by histology were much less differentiated in appearance than trichofolliculomas. A well circumscribed ring of highly mitotic, darkly staining basophilic cells with scant cytoplasm marked the borders of the tumors. These cells resembled hair matrix cells and were clearly distinct from basal epidermal and ORS cells. Their identity was confirmed by their expression of several markers of hair matrix cells, including *Lef-1*, *Shh* and *patched*. *Ptc* levels superseded those of surrounding follicles, a feature also seen in human basal cell carcinomas (Oro *et al.*, 1997). However, in contrast to basal cell carcinoma, centers of these less differentiated ΔN87βcat tumors were *Ptc* negative. In addition, the centers were filled with enucleated cellular ghosts, which bore a striking resemblance to "shadow" cells, the hallmarks of pilomatricomas, common hair matrix tumors in humans (Fitzpatrick *et al.*, 1993). As the ΔN87βcat phenotype mice develop two types of hair tumor, they can be used a model for human trichofolliculoma and pilomatricoma formation and serve as a screen for compounds that prevent the formation of such tumors as well as a screen for compounds that inhibit or suppress the Wnt signaling pathway.

Clinical use of the delivery of β-catenin activity will require that such activity is delivered and/or expressed transiently so as to obviate potential stimulation of tumor growth by long term delivery of β-catenin activity. There are a number of ways in which β-catenin activity can be delivered in a transient and/or tightly regulated fashion. Use of many conventional gene delivery approaches have failed to uniformly sustain transgene expression in genetically engineered skin past 4 weeks (Lu *et al.*, 1997; Fenjves *et al.*, 1996; Gerard *et al.*, 1993; Setoguchi *et al.*, 1994; Choate and Khavari, 1997). The present invention envisages taking advantage of the tendency for

transgenes to be expressed transiently in skin. In addition, inducible promoters, such as steroid-inducible promoters (White, 1997) can be used to drive expression of β-catenin transgenes. Controlled delivery of the inducing agent *in vivo* will result in concomitant controlled expression of the β-catenin polypeptide. For non-gene therapy treatments, the delivery over a defined period of time of agents that stabilize or otherwise enhance the activity or expression of endogenous β-catenin, will result in the required regulated and transient delivery of β-catenin activity. An advantage of using β-catenin gene therapy, is that use of an endogenous protein should obviate immune reactions, a major problem in gene therapy.

Despite the skin hair-tumors, the transgenic ΔN87βcat phenotype mice appeared healthy and normally active as far as they were grown, up to one year. The bigger tumors are rare and appear only in a few animals at relatively "old age." Thus, even transgenic expression of ΔN87βcat in domestic animals could be used to enhance hair follicle formation.

F. Engineering Expression Constructs

In certain embodiments, the present invention involves the manipulation of genetic material to produce expression constructs that encode therapeutic β-catenin genes. Such methods involve the generation of expression constructs containing, for example, a heterologous DNA encoding a gene of interest and a means for its expression, replicating the vector in an appropriate helper cell, obtaining viral particles produced therefrom, and infecting cells with the recombinant virus particles. The invention also envisages the use of multigene constructs wherein a β-catenin gene is administered with another hair growth or morphogenesis regulatory therapeutic gene.

1. β-Catenin and β-Catenin Gene

As used herein, the term "β-catenin" is intended to refer to a polypeptide based on the native sequence of β-catenin. This includes analogs and derivatives of wild-type β-catenin which retain the wild-type β-catenin's activity to induce hair

growth. In a preferred embodiment, the β-catenin derivative is ΔN87βcat, which is a N-terminally truncated β-catenin mutant. A "β-catenin gene" is a polynucleotide that encode a "β-catenin" polypeptide."

Amino acid sequence variants of the polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate

molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. Table 1 shows the codons that encode particular amino acids.

TABLE 1

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine

(-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of a immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

Another aspect of the present invention includes novel compositions comprising isolated and purified β-catenin-derived peptides, synthetic modifications of these, epitopic peptides, peptides derived from site-specifically-mutagenized nucleic acid segments encoding such peptides.

Mutagenesis may be performed in accordance with any of the techniques known in the art such as and not limited to synthesizing an oligonucleotide having one or more mutations within the sequence of a particular protein. In particular, site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 15, or 16, or 17 or 18 nucleotides, or even up to and including about 25, or 35, or 45, or even about 75 nucleotides or more in length is preferred, with about 14 to about 20 or 30 or more residues being highly preferred. Typically, about 8 or 9 or 10 or so unmodified nucleotides will flank the particular sequence being altered, which itself may be 10 or 15, or 20, or even 25-35 or so nucleotides in length.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence

is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement. A genetic selection scheme was devised by Kunkel *et al.* (1987) to enrich for clones incorporating the mutagenic oligonucleotide.

Alternatively, the use of PCR™ with commercially available thermostable enzymes such as *Taq* polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCR™-mediated mutagenesis procedures of Tomic *et al.* (1990) and Upender *et al.* (1995) provide two examples of such protocols. A PCR™ employing a thermostable ligase in addition to a thermostable polymerase may also be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector. The mutagenesis procedure described by Michael (1994) provides an example of one such protocol.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its

initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing. Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent 4,237,224, specifically incorporated herein by reference in its entirety.

Codons are selected from a preferred codon table so as to avoid codons which are rarely found in β-catenin genomes. Regions with many consecutive A+T bases or G+C bases are predicted to have a higher likelihood to form hairpin structures due to self-complementary. Disruption of these regions by the insertion of heterogeneous base pairs is preferred and should reduce the likelihood of the formation of self-complementary secondary structures such as hairpins which are known in some organisms to inhibit transcription (transcriptional terminators) and translation (attenuators). Alternatively, a completely synthetic gene for a given amino acid sequence can be prepared. Restriction sites found in commonly used cloning vectors are preferably avoided. However, placement of several unique restriction sites throughout the gene is useful for analysis of gene expression or construction of gene variants.

2. Selectable Markers

In certain embodiments of the invention, the therapeutic expression constructs of the present invention contain nucleic acid constructs whose expression may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants. For example, genes that

confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Recently, Deng *et al.* (1998), has shown that blasticidin is superior to the more commonly used neomycin in the selection of transduced keratinocytes. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art and include reporters such as EGFP, β–gal or chloramphenicol acetyltransferase (CAT).

3. Control Regions

a. Promoters

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40

early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β-actin, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of

other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic. An indelible promoter may be used to control the expression of a β-catenin polypeptide. Topical or other application of the inducing compound *in vivo* can be used to regulate the expression of β-catenin activity. Steroid indelible promoters (White, 1997) may be used for this purpose.

The ecdysone system (Invitrogen, Carlsbad, CA) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of Drosophila, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constituitively expressed from one vector, whereas the ecdysone-responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would then allow for the production of the gene transfer

vector without expression of a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristeron A.

Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, 1992; Gossen *et al.*, 1995). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-On™ system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-Off™ system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli*. The tetracycline operator sequence to which the tetracycline repressor binds, and the tetracycline repressor protein. The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-Off™ system, of the VP16 domain from the herpes simplex virus and the wild-type tertracycline repressor. Thus in the absence of doxycycline, transcription is constituitively on. In the Tet-On™ system, the tetracycline repressor is not wild type and in the presence of doxycycline activates transcription. For gene therapy vector production, the Tet-Off™ system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constituitively on.

In some circumstances, it may be desirable to regulate expression of a transgene in a gene therapy vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter if often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoetic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used.

Other viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

The invention provides embodiments wherein skin tissue specific promoters are used to effect β-catenin transcription in epidermal cells. Skin specific promoters include the keratin promoters including, but not limited to, K3, K5, K10, K14, K16. (Byrne *el al.*, 1994, Wang *et al.*, 1997, Page and Brownlee, 1998; Blumberg *et al.*, 1992). In preferred embodiments of the invention K5 and K14 promoters are used. The keratins K5 and K14 are major proteins expressed by mitotically active cells of the epidermis and epidermal appendages. The genes encoding K5 and K14 are abundantly transcribed in cultured keratinocytes (Stellmach *et al.*, 1991). In transient transfections, the K14 promoter directs the expression of a luciferase reporter in keratinocytes much more potently than in breast cancer cells. (Staggers *et al.*, 1995). In another embodiment of the invention, hair specific ultra-high-sulfur keratin promoters are used. (McNab *et al.*, 1990; Damak *et al.*, 1996a). This list of skin specific promoters should not be construed to be exhaustive or limiting, those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

b. Enhancers

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are

often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 2 and Table 3). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 2

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase

Table 2 – Continued

Albumin Gene
α-Fetoprotein
τ-Globin
β-Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 3

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

c. Polyadenylation Signals

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an

element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

4. Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene polycistronic messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated, Cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker. IRES sequences have been used to maximize the gene-transfer efficiency for skin gene therapy (Deng *et al.*, 1998).

G. Viral Vectors

In preferred embodiments of the invention, the expression construct comprise a virus or engineered construct derived form a viral genome. It is contemplated that a variety of viral particles may be employed according to the present invention. To determine whether other viral vectors could be manipulated in this manner, one of skill in the art can perform simple molecular biology techniques and assays. For example, replacement of promoter elements can be achieved through various cloning

techniques well known in the art. Assays for the detection of gene expression such as Northern or Western blots can then be used to determine expression levels of the viral gene products in various cell types, either *in vitro*, or *in vivo*, through the use of biopsies. Similarly, techniques to measure both the humoral and cell-mediated immune response to antigens are well known in the art and include enzyme-linked immunosorbent assays (ELISA), cytotoxic T Lymphocyte (CTL) assays and natural killer cell assays. *In vitro* infectivity assays also measure cytotoxic or cytopathic effects. Alternatively, viral vectors with a marker gene, such as β-galactosidase or luciferase as the transgene, can be employed. Measurement of the expression of the marker gene can then be done either qualitatively (*e.g.*, microscopically) or quantitatively (*e.g.*, flow cytometrically).

I. Adenovirus

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized DNA genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. The roughly 36 kB viral genome is bounded by 100-200 base pair (bp) inverted terminal repeats (ITR), in which are contained *cis*-acting elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome that contain different transcription units are divided by the onset of viral DNA replication.

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes (L1, L2, L3, L4 and L5), including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 map units) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5′ tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In order for adenovirus to be optimized for gene therapy, it is necessary to maximize the carrying capacity so that large segments of DNA can be included. It also is very desirable to reduce the toxicity and immunologic reaction associated with certain adenoviral products. The two goals are, to an extent, coterminous in that elimination of adenoviral genes serves both ends. By practice of the present invention, it is possible achieve both these goals while retaining the ability to manipulate the therapeutic constructs with relative ease.

The large displacement of DNA is possible because the *cis* elements required for viral DNA replication all are localized in the inverted terminal repeats (ITR) (100-200 bp) at either end of the linear viral genome. Plasmids containing ITR's can replicate in the presence of a non-defective adenovirus (Hay *et al.*, 1984). Therefore, inclusion of these elements in an adenoviral vector should permit replication.

In addition, the packaging signal for viral encapsidation is localized between 194-385 bp (0.5-1.1 map units) at the left end of the viral genome (Hearing *et al.*, 1987). This signal mimics the protein recognition site in bacteriophage λ DNA where a specific sequence close to the left end, but outside the cohesive end sequence, mediates the binding to proteins that are required for insertion of the DNA into the head structure. E1 substitution vectors of Ad have demonstrated that a 450 bp (0-1.25 map units) fragment at the left end of the viral genome could direct packaging in 293 cells (Levrero *et al.*, 1991).

Previously, it has been shown that certain regions of the adenoviral genome can be incorporated into the genome of mammalian cells and the genes encoded thereby expressed. These cell lines are capable of supporting the replication of an adenoviral vector that is deficient in the adenoviral function encoded by the cell line. There also have been reports of complementation of replication deficient adenoviral vectors by "helping" vectors, *e.g.*, wild-type virus or conditionally defective mutants.

Replication-deficient adenoviral vectors can be complemented, in *trans*, by helper virus. This observation alone does not permit isolation of the

replication-deficient vectors, however, since the presence of helper virus, needed to provide replicative functions, would contaminate any preparation. Thus, an additional element was needed that would add specificity to the replication and/or packaging of the replication-deficient vector. That element, as provided for in the present invention, derives from the packaging function of adenovirus.

It has been shown that a packaging signal for adenovirus exists in the left end of the conventional adenovirus map (Tibbetts, 1977). Later studies showed that a mutant with a deletion in the E1A (194-358 bp) region of the genome grew poorly even in a cell line that complemented the early (E1A) function (Hearing and Shenk, 1983). When a compensating adenoviral DNA (0-353 bp) was recombined into the right end of the mutant, the virus was packaged normally. Further mutational analysis identified a short, repeated, position-dependent element in the left end of the Ad5 genome. One copy of the repeat was found to be sufficient for efficient packaging if present at either end of the genome, but not when moved towards the interior of the Ad5 DNA molecule (Hearing *et al.*, 1987).

By using mutated versions of the packaging signal, it is possible to create helper viruses that are packaged with varying efficiencies. Typically, the mutations are point mutations or deletions. When helper viruses with low efficiency packaging are grown in helper cells, the virus is packaged, albeit at reduced rates compared to wild-type virus, thereby permitting propagation of the helper. When these helper viruses are grown in cells along with virus that contains wild-type packaging signals, however, the wild-type packaging signals are recognized preferentially over the mutated versions. Given a limiting amount of packaging factor, the virus containing the wild-type signals are packaged selectively when compared to the helpers. If the preference is great enough, stocks approaching homogeneity should be achieved.

First generation adenovirus vectors contain deletions in the E1 region, and the replication of these defective vectors is supported by packaging cell lines such as 293 cells that provide the E1 region gene products. Similarly, adenovirus vectors with the E1 and E4 gene deleted, but provided by 293 cells expressing both viral gene

products, have been made. It is possible to make even larger deletions on the adenovirus genome and then provide the deleted genes in trans, either by a helper virus or helper cell, or both.

The ability to selectively reduce the level of viral gene expression also makes adenovirus desirable as a gene transfer vector. The complete removal of these adenoviral genes would obviously eliminate the host immune response, however, it can be difficult to establish packaging cell lines because of viral gene-mediated toxicity. The replacement of viral promoter elements with a synthetic promoter, significantly reduces the level of viral gene expression.

Adenoviral vectors with synthetic promoters will have at least i) a deletion of the E1B and/or E1A region and ii) an inducible or synthetic promoter substituted for the normal viral promoter that regulates expression of one or more of the early adenoviral genes E2, E3, E4 and E5. Similarly, a synthetic promoter could be substituted for the adenoviral major late promoter, which regulates expression of the late adenoviral gene L1, L2, L3, L4 and L5. Any vector with at least one promoter replacement is envisioned. For example, combinations of early region replacement include E1A and E1B, E1A and E2, E1B and E2, E1A and E4, E1B and E4, E1A and E5, E1B and E5, E1A and E1B and E2, E1A and E1B and E4, E1A and E1B and E5, E1A and E1B and E2 and E4, E1A and E1B and E2 and E5, E1A and E1B and E4 and E5, and E1A and E1B and E2 and E4 and E5.

To further reduce the expression of viral proteins in an adenoviral vector, one or more inducible or synthetic promoters, either of the same or different origin, may be used together to regulate transcription of viral genes. For example, in an E1 region-deleted adenovirus, the GAL4/TATA promoter may be substituted for the adenoviral E4 promoter, as well as the adenoviral E2 promoter, so that expression of the E2 and E4 genes is only possible in producer cells expressing the GAL4/VP16 transactivating polypeptide. Similarly, the GAL4/TATA promoter could be substituted for the E2 promoter, and the GAL4-estrogen receptor response element promoter could be linked to the E4 gene. In such a system (see below), the producer

cells would express the GAL4/VP16 fusion polypeptide to drive expression of the E2 gene, as well as the GAL4/ER (estrogen receptor) fusion polypeptide to drive expression of the E4 gene. Any number of synthetic or inducible promoter combinations could be substituted for one or more of the viral gene promoters, thus retaining precise control over the viral gene expression and replication.

2. Retrovirus

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - gag, pol and env - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Ψ, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and also are required for integration in the host cell genome (Coffin, 1990). Conditions for the efficient transduction of early passage human keratinocytes by retroviral vectors have recently been described (Deng., *et al* 1998).

In order to construct a retroviral vector, a nucleic acid encoding a promoter is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol and env genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to

infect a broad variety of cell types. However, integration and stable expression of many types of retroviruses require the division of host cells (Paskind *et al.*, 1975).

An approach designed to allow specific targeting of retroviral vectors recently was developed based on the chemical modification of a retrovirus by the chemical addition of galactose residues to the viral envelope. This modification could permit the specific infection of cells *via* asialoglycoprotein receptors, should this be desired.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, the infection of a variety of human cells that bore those surface antigens was demonstrated with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

Deng *et al.* (1997) have described methods for to sustain retroviral-induced transgene expression in keratinocytes. Conventional viral vectors tends to fail to uniformly sustain transgene expression in skin past 1 to 4 weeks. This is the period spanning the time for epidermal turnover. Methylation of the retroviral long-term repeat (LTR) is implicated as the mechanism of loss of retroviral gene expression (Chakraborty *et al.*, 1993). Deng *et al.* (1997) describe the deletion and of promoter and enhancer sequences at the 3' LTR, to produce self-inactivating retroviral vectors. The 5' LTR ultimately replaces the deleted 3' LTR and the vector is transcriptionally inactivated. An internal promoter is used to drive gene expression. Transgene expression in genetically engineered skin resulting from use of such vectors persisted longer as compared to conventional retroviral vectors.

3. *Adeno-associated Virus*

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three

different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription.

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

The terminal repeats of the AAV vector can be obtained by restriction endonuclease digestion of AAV or a plasmid such as p201, which contains a modified AAV genome (Samulski *et al.* 1987), or by other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis of the terminal repeats based upon the published sequence of AAV. The ordinarily skilled artisan can determine, by well-known methods such as deletion analysis, the minimum sequence or part of the AAV ITRs which is required to allow function, *i.e.*, stable and site-specific integration. The ordinarily skilled artisan also can determine which minor modifications of the sequence can be tolerated while maintaining the ability of the terminal repeats to direct stable, site-specific integration.

AAV-based vectors have proven to be safe and effective vehicles for gene delivery *in vitro,* and these vectors are being developed and tested in pre-clinical and

clinical stages for a wide range of applications in potential gene therapy, both *ex vivo and in vivo* (Carter and Flotte, 1996 ; Chatterjee *et al.*, 1995; Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Flotte *et al.*, 1993; Goodman *et al.*, 1994; Kaplitt *et al.*, 1994; 1996, Kessler *et al.*, 1996; Koeberl *et al.*, 1997; Mizukami *et al.*, 1996; Xiao *et al.*, 1996).

AAV-mediated efficient gene transfer and expression in the lung has led to clinical trials for the treatment of cystic fibrosis (Carter and Flotte, 1996; Flotte *et al.*, 1993). Similarly, the prospects for treatment of muscular dystrophy by AAV-mediated gene delivery of the dystrophin gene to skeletal muscle, of Parkinson's disease by tyrosine hydroxylase gene delivery to the brain, of hemophilia B by Factor IX gene delivery to the liver, and potentially of myocardial infarction by vascular endothelial growth factor gene to the heart, appear promising since AAV-mediated transgene expression in these organs has recently been shown to be highly efficient (Fisher *et al.*, 1996; Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; 1996; Koeberl *et al.*, 1997; McCown *et al.*, 1996; Ping *et al.*, 1996; Xiao *et al.*, 1996).

4. Herpesvirus

Because herpes simplex virus (HSV) is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating in to the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency makes HSV an attractive vector. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues given its wide host range.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, *etc.*) makes it possible to control expression to a greater extent

than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings. For a review of HSV as a gene therapy vector, see Glorioso *et al.* (1995).

HSV, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. The large, complex, double-stranded DNA genome encodes for dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman 1975; Roizman and Sears, 1995). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by the virion protein number 16, or α-transinducing factor (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell, *et al.*, 1984). The expression of β genes requires functional α gene products, most notably ICP4, which is encoded by the α4 gene (DeLuca *et al.*, 1985). γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Holland *et al.*, 1980).

In line with the complexity of the genome, the life cycle of HSV is quite involved. In addition to the lytic cycle, which results in synthesis of virus particles and, eventually, cell death, the virus has the capability to enter a latent state in which the genome is maintained in neural ganglia until some as of yet undefined signal triggers a recurrence of the lytic cycle. Avirulent variants of HSV have been

developed and are readily available for use in gene therapy contexts (U.S. Patent No. 5,672,344).

5. Vaccinia Virus

Vaccinia virus vectors have been used extensively because of the ease of their construction, relatively high levels of expression obtained, wide host range and large capacity for carrying DNA. Vaccinia contains a linear, double-stranded DNA genome of about 186 kb that exhibits a marked "A-T" preference. Inverted terminal repeats of about 10.5 kb flank the genome. The majority of essential genes appear to map within the central region, which is most highly conserved among poxviruses. Estimated open reading frames in vaccinia virus number from 150 to 200. Although both strands are coding, extensive overlap of reading frames is not common.

At least 25 kb can be inserted into the vaccinia virus genome (Smith and Moss, 1983). Prototypical vaccinia vectors contain transgenes inserted into the viral thymidine kinase gene *via* homologous recombination. Vectors are selected on the basis of a tk-phenotype. Inclusion of the untranslated leader sequence of encephalomyocarditis virus, the level of expression is higher than that of conventional vectors, with the transgenes accumulating at 10% or more of the infected cell's protein in 24 h (Elroy-Stein *et al.*, 1989).

6. SV40 Virus

Simian virus 40 (SV40) was discovered in 1960 as a contaminant in polio vaccines prepared from rhesus monkey kidney cell cultures. It was found to cause tumors when injected into newborn hamsters. The genome is a double-stranded, circular DNA of about 5000 bases encoding large (708 AA) and small T antigens (174 AA), agnoprotein and the structural proteins VP1, VP2 and VP3. The respective size of these molecules is 362, 352 and 234 amino acids.

Little is known of the nature of the receptors for any polyoma virus. The virus is taken up by endocytosis and transported to the nucleus where uncoating takes place. Early mRNA's initiate viral replication and is necessary, along with DNA replication,

for late gene expression. Near the origin of replication, promoters are located for early and late transcription. Twenty-one base pair repeats, located 40-103 nucleotides upstream of the initiation transcription site, are the main promoting element and are binding sites for Sp1, while 72 base pair repeats act as enhancers.

Large T antigen, one of the early proteins, plays a critical role in replication and late gene expression and is modified in a number of ways, including N-terminal acetylation, phosphorylation, poly-ADP ribosylation, glycosylation and acylation. The other T antigen is produced by splicing of the large T transcript. The corresponding small T protein is not strictly required for infection, but it plays a role in the accumulation of viral DNA.

DNA replication is controlled, to an extent, by a genetically defined core region that includes the viral origin of replication. The SV40 element is about 66 bp in length and has subsequences of AT motifs, GC motifs and an inverted repeat of 14 bp on the early gene side. Large T antigen is required for initiation of DNA replication, and this protein has been shown to bind in the vicinity of the origin. It also has ATPase, adenylating and helicase activities.

After viral replication begins, late region expression initiates. The transcripts are overlapping and, in some respect, reflect different reading frames (VP1 and VP2/3). Late expression initiates is the same general region as early expression, but in the opposite direction. The virion proteins are synthesized in the cytoplasm and transported to the nucleus where they enter as a complex. Virion assembly also takes place in the nucleus, followed by lysis and release of the infectious virus particles.

It is contemplated that the present invention will encompass SV40 vectors lacking all coding sequences. The region from about 5165-5243 and about 0-325 contains all of the control elements necessary for replication and packaging of the vector and expression of any included genes. Thus, minimal SV40 vectors are derived from this region and contain at least a complete origin of replication.

Because large T antigen is believed to be involved in the expression of late genes, and no large T antigen is expressed in the target cell, it will be desired that the promoter driving the heterologous gene be a polyomavirus early promoter, or more preferably, a heterologous promoter. Thus, where heterologous control elements are utilized, the SV40 promoter and enhancer elements are dispensable.

7. Other Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as lentivirus, poxvirus, alphavirus and coxsackie virus. These viruses offer several features for use in gene transfer into various mammalian cells.

H. Non-viral Transfer

DNA constructs of the present invention are generally delivered to a cell, in certain situations, the nucleic acid to be transferred is non-infectious, and can be transferred using non-viral methods.

Several non-viral methods for the *in vivo* and *ex vivo* transfer of expression constructs into skin cells are contemplated by the present invention. These include calcium phosphate precipitation (Jiang *et al.*, 1991; Graham and Van Der Eb. 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Jiang *et al.*. 1991; Gopal, 1985), electroporation (Jiang *et al.*, 1991; Fenjves, 1994; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Fenjves, 1994; Harland and Weintraub, 1985), DNA-loaded liposomes (Jiang *et al.*, 1991; Fenjves, 1994; Nicolau and Sene, 1982; Fraley *et al.*, 1979), cationic lipid-DNA lipoplexes (Escriou *et al.*, 1998a, 1998b; Zelphati *et al.*, 1996, 1998; Dodds *et al.*, 1998), cationic polymer-DNA complexes (Goldman *et al.*, 1997; Abdallah *et al.*, 1996; Ferrari *et al.*, 1997), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

Once the construct has been delivered into the cell the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991a)

Liposome-mediated nucleic acid delivery and expression of foreign DNA, "lipofection," has been very successful *in vitro*. Lipofection is an efficient system of DNA delivery to primary keratinocytes (Jaing *et al.*, 1991). Liposomes may also be used for the *in vivo* topical application of cutaneous gene therapies. Topical application of liposomes has been successfully used to target the delivery of a retroviral vector to cells in the hair follicle (Li and Hoffman, 1995; Hoffman 1998). Sawamura *et al*, (1997) have used a hemagglutinating virus of Japan (HVJ)-liposome combination for the topical delivery of DNA. This method involves the entrapment of DNA and nuclear protein within liposomes and the use of HJV to enhance liposome fusion with cell membranes (Kaneda *et al.*, 1989). Alexander and Akhurst (1995), showed that, utilizing a commercially available liposomes preparation, topically

applied DNA-liposomes complexes can be efficiently delivered and expressed in skin cells.

In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules (Radler *et al.*, 1997). The structures formed by cationic lipids and DNA are more variable and complex than simple liposomes and have been termed lipoplexes (Flegner, 1997). Lipoplexes have been widely used as gene transfer vectors (Escriou *et al* 1998a, 1998b; Zelphati *et al.*, 1996, 1998; Dodds *et al.*, 1998). A cationic lipid vector has been used in the Phase I study of direct gene transfer of HLA-B7 in patients with metastatic melanoma (Stopeck *et al.*, 1997).

In other embodiments, cationic polymer-DNA complexes can be used as delivery vehicles for DNA. Simple cationic polymers will bind to DNA and assemble into discrete complexes (Wolfert *et al.*, 1996). Cationic-hydrophilic block copolymers can be used to increase aqueous solubility (Toncheva *et al.*, 1998). DNA-cationic polymer complexes have been widely used as synthetic vectors for delivery of genes *in vitro* and *in vivo* (Wagner *et al.*, 1991; Trubetskoy *et al.*, 1992; Pocet *et al.*, 1996; Coll *et al.*, 1997; Goldman *et al.*, 1997; Abdallah *et al.*, 1996; Ferrari *et al.*, 1997). U.S. Patent 5,679,559 discloses the use of a lipoprotein containing cationic polymer system for gene delivery.

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated

endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferring (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Naked DNA is internalized and expressed in skin after *in vivo* intradermal injection. (Hengge *et al.*, 1996). Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that

direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a β-catenin polypeptide may also be transferred in a similar manner *in vivo* and express β-catenin polypeptide.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads. The ballistic approach has been employed *in vivo* to achieve expression in skin (Cheng., *et al.*, 1993)

I. Pharmaceutical Vehicles and Routes of Administration

Pharmaceutical compositions of the present invention will have an effective amount of agent to induce hair growth. Administration of therapeutic compositions according to the present invention will be *via* any route so long as the target tissue is available *via* that route. A preferred route of administration are topical and intradermal injection. Alternatively, administration may be subcutaneous, ballistic, intramuscular, intraperitoneal or intravenous injection. Such compositions generally will be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated.

In a preferred embodiment, the therapeutic formulations of the invention are prepared in forms suitable for topical administration, such as cremes and lotions. The topical application of viral vectors, plasmid DNA and naked DNA has successfully resulted in the transfection of skin cells (Lu *et al.*, 1997; Li and Hoffman, 1995; Hoffman 1998; Sawamura *et al.*, 1997; Greenhalgh *et al.*, 1994; Alexander and Akhurst, 1995). To maximize infectivity it may be necessary to remove the superficial layers of the epidermis by tape stripping (Greenhalgh *et al.*, 1994). Alternatively, iontophoresis and related methods utilizing electric fields may be used to deliver the gene therapy into the skin (Hui, 1998; Zhang *et al.*, 1996). Other chemical and biophysical methods may be employed to enhance the bioavailability of topically applied therapeutic agents to enhance β-catenin-mediated hair growth.

The active compounds of the present invention may be formulated for parenteral administration, for example, formulated for injection *via* the intradermal, subcutaneous intravenous routes. The preparation of an aqueous composition that contains an effective amount of therapeutic agents to induce hair growth will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can be emulsified.

In addition to the compounds formulated for parental administration, such as those for intradermal or intravenous injection, other pharmaceutically acceptable forms include tablets or other solids for oral administration; time release capsules; and any other form currently used.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydoxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

For the application of a gene therapy, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, including the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethlyamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens,

chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the composition of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously-sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

For parental administration in an aqueous solution, for example, the solution should be suitably buffered and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intradermal, subcutaneous or intravenous administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 100 ml of hypodermocylsis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some

variations in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In certain embodiments, the inventors contemplate the use of liposomes for the topical application or intradermal injection of one or more of the disclosed pharmaceutical composition into a host cell. Such formulations may be preferred for the introduction of pharmaceutically-acceptable formulations of β-catenin or β-catenin derivatives or analogs disclosed herein.

The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977 which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy of intracellular bacterial infections and diseases). More recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Chonn, 1987).

In one instance, the disclosed composition may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. The term "liposome" is intended to mean a composition arising spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991b).

In certain embodiments of the present invention, gene gun-mediated skin transfection will be used as the route of administration (Rakhmilevich *et al.*, 1996; Cheng *et al.*, 1993). This method involves coating DNA to gold particles directly into the skin at high velocity

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of import is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be a single administration, as for example a single injection, but may comprise continuous administration over a set period of time, as for example a continuos perfusion. For gene therapies, unit doses of the present invention may conveniently may be described in terms of plaque forming units (pfu) of the viral construct. Unit doses range from 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} pfu and higher.

The invention provides for the delivery of β-catenin activity such that the resulting level of β-catenin polypeptide in an epidermal or ORS cell is 2-100 fold higher than level of endogenous β-catenin in an equivalent untreated epidermal or ORS cell. Expression of a β-catenin polypeptide can be established by Westen blot and quantiifed by ELISA and raidoimmunoassays. The delivery of an effective dose of β-catenin activity will be that sufficient to cause translocation of *Lef-1* to the nucleus of an epidermal cell. The translocation of *Lef-1* can be monitored by immunohistochemical methodologies.

In certain embodiments the invention provides for the *ex vivo* gene delivery. Skin as a tissue is ideally suited to *ex vivo* gene therapy treatments (Wang *et al.*, 1997). The accesibility of skin and the proliferative capacity of cultred epidermal cells makes keratinocytes ideal candiaites for transfection and gene therapy. Thus a patient's skin cells are cultured, transduced *in vitro*, then returned to the patient. Large number of skin cells can be obtained from a small skin biopsy and the cell culturing conditions are well defined (Reinwald and Green, 1975). Cultured keratinocytes form an epithelial sheet when approaching confluence and can be as an autologous skin graft (Greenhalgh *et al.*, 1994). Alternatively, transduced cells can be injected intradermally into the patient's skin. Prior to return to the patient, keratinocytes can

be screened for the effective delivery of β-catenin activity by monitoring for the translocation of *Lef-1* to the nucleus. If an inducible promoter, such as a steroid inducible promoter (White, 1997), is used, the effective delivery of β-catenin activity *in vitro* can be established by monitoring for nuclear translocation of *Lef-1* after exposure of transfected keratinocytes to the inducing agent. Once the keratinocytes are returned to the patient, *in vivo* delivery of β-catenin activity can be initiated by delivery of the inducing agent by topical or other routes of delivery.

J. DIAGNOSTIC AND SCREENING TESTS

Methods for screening for β-catenin expression and responsiveness of skin cells to β-catenin are another aspect of the present invention. These parameters may also be predicative of the propensity to certain types of hair loss. In addition, these methods can be used to screen compounds for the ability to deliver or induce β-catenin activty. The translocation of *Lef*-1 to the nucleus is indicative of the effective delivery of β-catenin activty and can be used as the basis for a high-through-put screening methodologies for investigating candidate compounds for the ability to deliver or induce β-catenin activty. Such screening may be facilitated by the use of the reporter construct TOPFLASH. This reporter contains the luciferase gene under the control of an enhancer containing *Lef-1*/Tcf elements. Thus, however induced, nuclear β-catenin together with *Lef-1* will activate the reporter which is readily monitored by a luminometer. This assay is readily adaptable for large scale screening using, for example, microtiter plate assay methodologies. The same enhancer linked to the GFP protein may also be used for screening.

1. Immunodectection Methods

The present invention concerns immunodetection methods for binding, quantifying or otherwise generally detecting β-catenin and Lef-1/Tcf protein components. The β-catenin Lef-1/Tcf antibodies prepared in accordance with the present invention may be employed to detect wild-type or mutant β-catenin proteins or peptides. As described throughout the present application, the use of wild-type or mutant specific antibodies is contemplated. The steps of various useful

immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura *et al.* (1987), incorporated herein by reference.

In general, the immunobinding methods include obtaining a sample suspected of containing a β-catenin or Lef-1/Tcf protein or peptide, and contacting the sample with a first antiβ-catenin or antiLef1/Tcf antibody in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

These methods include methods for purifying wild-type or mutant proteins or peptides as may be employed in purifying wild-type or mutant proteins or peptides from patients' samples or for purifying recombinantly expressed wild-type or mutant β-catenin proteins or peptides. In these instances, the antibody removes the antigenic wild-type or mutant β-catenin protein or peptide component from a sample. The antibody will preferably be linked to a solid support, such as in the form of a column matrix, and the sample suspected of containing the wild-type or mutant protein antigenic component will be applied to the immobilized antibody. The unwanted components will be washed from the column, leaving the antigen immunocomplexed to the immobilized antibody, which wild-type or mutant protein antigen is then collected by removing the wild-type or mutant protein or peptide from the column.

The immunobinding methods also include methods for detecting or quantifying the amount of a wild-type or mutant protein reactive component in a sample, which methods require the detection or quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing a wild-type or mutant protein or peptide, and contact the sample with an antibody against wild-type or mutant and then detect or quantify the amount of immune complexes formed under the specific conditions.

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing a wild-type or mutant protein-specific antigen, such as a cancer tissue section or specimen, a homogenized cancer tissue extract, a

cancer cell, separated or purified forms of any of the above wild-type or mutant protein-containing compositions, or even any biological fluid that comes into contact with cancer tissue, including blood and serum, although tissue samples and extracts are preferred.

Contacting the chosen biological sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time lone enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any wild-type or mutant β-catenin protein antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological or enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The wild-type or mutant antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody,

which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

The immunodetection methods of the present invention have evident utility in the diagnosis or prognosis of conditions such as various forms of cancer. Here, a biological or clinical sample suspected of containing a wild-type or mutant protein, peptide or mutant is used. However, these embodiments also have applications to non-clinical samples, such as in the titering of antigen or antibody samples, in the selection of hybridomas, and the like.

In the clinical diagnosis or monitoring of patients with various forms of cancer, the detection of a β-catenin mutant, or an alteration in the levels of β-catenin in comparison to the levels in a corresponding biological sample from a normal subject is indicative of a patient with cancer. However, as is known to those of skill in the art, such a clinical diagnosis would not necessarily be made on the basis of this method in isolation. Those of skill in the art are very familiar with differentiating between significant differences in types or amounts of biomarkers, which represent a positive identification, and low level or background changes of biomarkers. Indeed,

background expression levels are often used to form a "cut-off" above which increased detection will be scored as significant or positive.

a. ELISAs

As detailed above, immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and Western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, the anti-wild-type or mutant antibodies of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the wild-type or mutant protein antigen, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound wild-type or mutant β-catenin protein antigen may be detected. Detection is generally achieved by the addition of another anti-wild-type or mutant antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second anti-wild-type or mutant antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples suspected of containing the wild-type or mutant protein antigen are immobilized onto the well surface and then contacted with the anti-wild-type or mutant β-catenin antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound anti-wild-type or mutant antibodies are detected. Where the initial anti-wild-type or mutant antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected

using a second antibody that has binding affinity for the first anti-wild-type or mutant antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the wild-type or mutant proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies against wild-type or mutant protein are added to the wells, allowed to bind, and detected by means of their label. The amount of wild-type or mutant protein antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies against wild-type or mutant polypeptide or protein before or during incubation with coated wells. The presence of wild-type or mutant protein in the sample acts to reduce the amount of antibody against wild-type or mutant protein available for binding to the well and thus reduces the ultimate signal. This is also appropriate for detecting antibodies against wild-type or mutant protein in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described below.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or

antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions

that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

b. Immunohistochemistry

The antibodies of the present invention may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). For example, each tissue block consists of 50 mg of residual "pulverized" diabetic tissue. The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" diabetic tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block

from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

2. Nucleic Acids

Expression of β-catenin RNA can be measured by RT-PCR™. Nucleic acids used as a template for amplification and detection are isolated from cells contained in biological samples according to standard methodologies (Sambrook *et al.*, 1989). Where RNA is used, it may be desired to convert the RNA to a complementary DNA.

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641, filed December 21, 1990, incorporated herein by reference. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a

single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA

(dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M.A., In: *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990 incorporated by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the

membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

All the essential materials and reagents required for detecting β-catenin protein markers in a biological sample may be assembled together in a kit. This generally will comprise preselected primers for specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, *etc.*), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

Such kits generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair.

K. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Material and Methods

Plasmids. The pK14ΔN87βcat vector was constructed in two steps: (1) The sense primer included a *Bam*HI site, an ideal Kozak sequence and nucleotides 462-482 of human β-catenin cDNA (obtained from Dr. Birchmeier, Univ. Berlin, Germany); the anti-sense primer included nucleotides 2550-2532 of human β-catenin cDNA and a *Bam*HI site. These were used with human β-catenin cDNA as template to synthesize a PCR™ product encoding an 87 amino acid N-terminally truncated β-catenin. This fragment was subcloned into the *Bam*HI site of the K14 expression vector. (2) The *Xho*I-*Bgl*II fragment of the resulting plasmid, containing most of the β-catenin insert (1996 bp) was replaced with the original β-catenin cDNA, and all PCR™/primer-engineered segments were verified by sequencing. The vector pCMV-hLef1 encodes the full length hLef-1 product (Zhou *et al.*, 1995). The luciferase reporter construct pTOPFLASH was obtained from Dr. van de Wetering and Dr. Clevers (University Hospital, Utrecht, The Netherlands).

Cell Culture and Transfection. A spontaneously arising keratinocyte cell line was isolated from an epidermal primary keratinocyte culture of murine skin. This line, termed UIMK, displays many of the characteristics of primary keratinocytes, including basal keratin expression and calcium-inducible differentiation (U. Gat, unpublished). Keratinocytes were cultivated as described (Hennings *et al*, 1980). Transfections were performed using the SuperFect polycationic reagent (Qiagen) or the lipid based FuGENE6 reagent (BMB) according to manufacturer's protocol except that the SuperFect-DNA complex was left on the plates for 6 h instead of 2. Cells transfected with pTOPFLASH were processed and assayed for luciferase activity with the luciferase assay system kit (Promega, Madison, WI) and a luminometer.

Generation of Transgenic Mice. The K14-ΔN87βcat transgene was isolated from the vector and injected into fertilized CD-1 mouse embryos and transgenic mice were generated as described previously (Vassar *et al.*, 1989). Mice positive for the

transgene were identified by PCR™ analysis of toe genomic DNA and transgene expression was determined by RT-PCR™.

Immunohistochemistry. Frozen sections of tissues or methanol fixed coverslips with transfected keratinocytes were subjected to double indirect immunostaining (Vassar and Fuchs, 1991). For analysis of fixed, paraffin embedded tissue, antigen unmasking was performed according to Manabe *et al.* (1996). Primary antibodies used were: mouse monoclonal anti-trichohyalin (AE15; Manabe *et al.*, 1996) or anti-hair keratin (AE13; Lynch *et al.*, 1986); and guinea pig anti-K5 (Byrne *et al.*, 1994). Fluorescence conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA).

In situ hybridization probes. Digoxygenin hybridization probes were synthesized according to the manufacturer's instructions (Boehringer Mannheim biochemicals, Indianapolis, IN). A 517-bp mouse Lef-1 cDNA was made by excising a 700-bp *Bam*HI fragment from the 1217-bp RT-PCR™ mouse Lef-1 cDNA clone (in pCRII) that was made as described (Zhou *et al.*, 1995). This procedure eliminated the HMG sequences conserved among family members. Antisense probe was made by linearizing the plasmid with *Eco*RV and transcribing with SP6 polymerase. Sense cRNA was made by linearizing with *Bam*HI and transcribing with T7 polymerase. A 2.8kb mouse *Shh* cDNA subcloned into pBlueScript KS+ (Johnson and Tabin, 1997) was used for expression studies. Antisense probe was made by linearizing plasmid with *Xba*I and transcribing with T7 RNA polymerase and the sense probe was made by linearizing with *Hin*dIII and transcribing with T3 polymerase. An 841-bp *Eco*RI fragment of mouse patched *(Ptc)* cDNA (Oro *et al.*, 1997) was subcloned into pBlueScriptII/KS+, and antisense probe was made by linearizing with *Bam*HI and transcribing with T3. Sense strand was made by linearizing with *Hin*dIII and labeling with T7 polymerase.

EXAMPLE 2

ΔN87βcat Synergizes With Lef-1 in Keratinocytes to Act As a Transcription Factor

Others have shown previously that N-terminally truncated forms of β-catenin accumulate in the cytoplasm of a number of cell types, including mammalian fibroblasts and simple epithelial cells, but are not impaired in their ability to bind E-cadherin, α-catenin and Tcf/Lef (Funuyama *et al.*, 1995; Munemitsu *et al.*, 1996; Wylie *et al.*, 1996; van de Wetering *et al.*, 1997; Fagotto *et al.*, 1998; Wong *et al.*, 1998). Using this information, the inventors designed an ΔN87βcat expression vector and began by verifying that its behavior in keratinocytes is similar to that described previously. Upon co-expression with a Lef-1 transgene, β-catenin concentrated in keratinocyte nuclei in addition to its normal sites of cell-cell adhesion. Additionally, ΔN87βcat/Lef-1 functioned efficiently in keratinocytes to transactivate >20X a luciferase reporter gene driven by an enhancer containing multiple Tcf/Lef binding sequences (FIG. 2). When expressed alone, ΔN87βcat still enhanced transcription, but to a lesser extent (FIG. 2) presumably due to the presence of low levels of endogenous Lef-1 in keratinocytes (Zhou *et al.*, 1995). Whether Lef-1 and/or other Tcf family members function with β-catenin to repress other promoters in keratinocytes is an intriguing question beyond the scope of the present study.

EXAMPLE 3

Expression of ΔN87βcat In the Epidermis and Follicle Outer Root Sheath of Mice

To drive ΔN87βcat expression in mice, the inventors used the K14 promoter, active in the mitotically active cells of the epidermis and the outer root sheath (ORS) of hair follicles (Vassar *et al.*, 1989; Byrne *et al.*, 1994; Wang *et al.*, 1997). This promoter has been tested extensively in conjunction with many different transgenes to give rise to highly restricted keratinocyte-specific expression in mice (Vassar *et al.*, 1989; Byrne *et al.*, 1994; Wang *et al.*, 1997; for review, see Fuchs, 1997). The transgene construct engineered is illustrated in FIG. 3.

The inventors first used polymerase chain reaction (PCR™) of tail DNAs to identify which mice carried the transgene, and RT-PCR™ to verify expression. The inventors verified keratinocyte-specific protein expression of ΔN87βcat by immunoblot analysis of proteins isolated from epidermis and dermis after their separation by 2M NaBr treatment of transgenic skin. An 80 kDa ΔN87βcat was detected by anti-β-catenin antibody in transgenic epidermis. This band was not seen in control epidermis or in transgenic dermis. The intensity of the ΔN87βcat was comparable to that of the endogenous 91 kDa β-catenin band. However, endogenous β-catenin was present ubiquitously throughout the skin epithelium, while the transgene was expressed only in the K14 promoter-active cells, constituting ~10-20% of this population (Vassar *et al.*, 1989; Wang *et al.*, 1997). Therefore, the inventors estimate that the level of ΔN87βcat is 5-10 fold higher than endogenous β-catenin in the basal epidermal and ORS follicle cells of the skin. This is consistent with the expected increased stability of the truncated version of β-catenin. Although the inventors were unable to detect nuclear ΔN87βcat, this was not unexpected given similar unsuccessful efforts in many cases where β-catenin is suspected to be nuclear (Willert and Nusse, 1998; Gumbiner *et al.*, 1998; Clevers and van de Wetering, 1997). Two independently derived lines behaved similarly in this and all subsequent analyses, thereby attributing the effects observed to transgene expression and not chromosomal integration site.

EXAMPLE 4

ΔN87βcat Mice Display Dramatic Changes in Skin and Hair Coat Only in Haired Skin Regions and Concomitant With the Initiation of the First Postnatal Hair Cycle

Despite strong transgene promoter activity by E14.5 (Byrne *et al.*, 1994; Wang *et al.*, 1997), an overt phenotype did not emerge until 24 days postnatally when mice of both lines began to display enlarged hind paws, which more than tripled in normal size. Homozygous adults developed thick ridges in their skin and hair coat, particularly around the ears, eyelids and nose. Several other abnormalities extended beyond the skin; a detailed description of these is beyond the scope of the present study and will be reported elsewhere.

Histological analyses revealed that the appearance of paw abnormalities coincided with initiation of the first postnatal hair cycle at d21. Prior to 18 days, only subtle differences were seen between wild-type and ΔN87βcat skin. At d18, most follicles in ΔN87βcat dorsal paw remained morphologically in the resting stage as in the control. Beginning at d18, unusual epithelial invaginations began to appear in the epidermis. Since each preexisting hair follicle still retained its original hair and since its orifice at the skin surface was clearly demarcated by this age, it was easy to see that these epithelial invaginations arose from interfollicular epidermis. Their number and size increased rapidly and by 21d, they bore an amazing resemblance to embryonic hair germs. Whereas preestablished hair follicles were always uniformly spaced in a highly patterned fashion, new invaginations were randomly positioned, and were often extremely close to one another.

Similar multiple outgrowths also arose radially from the permanent, but not the cycling, portion of the ORS; these were the prominent sites of backskin changes, which did not appear until several months after birth. Interestingly, below the sebaceous gland, each aberrant backskin follicle appeared to be morphologically normal. Additionally, follicles displaying a phenotype in their permanent segments appeared to be at early anagen, as judged by the short ORS stem between bulge and the matrix bulb (Hardy, 1992). Whether originating from interfollicular epidermis as in dorsal paw skin, or upper ORS as in backskin, invaginations quickly diverged from being epidermal/ORS-like to being hair matrix-like in morphology. Despite these remarkable changes, no changes were seen in ventral pawskin, devoid of hair follicles.

Whether interfollicular as in dorsal paw skin or upper ORS as in backskin, epithelial invaginations stemmed only from cells where K14 promoter activity was high. This said, K14 promoter activity is strong in the basal layer of the epidermis from unhaired as well as haired skin, and it is high throughout the epidermis and developing hair follicles by E14 of embryonic development. Thus, transgene expression alone was clearly not sufficient to account either for the regional or the temporal differences in the inductive process. Rather, whether in interfollicular

epidermis or in ORS, ΔN87βcat-mediated aberrations seemed to require some additional factor supplied only by haired skin, and which appeared only at specified times, appearing to coincide with hair cycle initiation. Taken together, these data raised the possibility that the as yet unidentified stimulus that is sensed normally by bulge cells to trigger the next hair growth cycle (Cotsarelis *et al.*, 1990; Wilson *et al.*, 1994) might now be sensed by ΔN87βcat-expressing cells, prompting them to proliferate and invaginate.

One obvious candidate for an internal change that could act in concert with ΔN87βcat to elicit a response is *Lef-1*. *In situ* hybridization revealed that despite the paucity of *Lef-1* mRNAs in most areas of the epidermis and ORS, they were found in the aberrant invaginations of both interfollicular epidermis and ORS. Expression was maintained at the base of these hair germ-like structures, similar to that seen in mature wild-type follicles. Addressing whether this induction is direct or indirect is an issue beyond the scope of the present study. However, the inventors' data demonstrate that some factor(s) present in haired skin at the start of hair growth cycles causes a change in ΔN87βcat-expressing cells that leads to their proliferation, invagination, and appearance of *Lef-1* mRNAs.

EXAMPLE 5

Development of Epithelioid Cysts and Trichofolliculomas in ΔN87βcat Skin: Evidence for Induction of *De Novo* Hair Morphogenesis

To quantitate the number of hair germ-like invaginations that appeared in ΔN87βcat skin, the inventors used d24 dorsal paw skin from control and transgenic mice. Nearly all epidermal segments between preestablished follicles displayed at least one hair germ-like invagination. Some interfollicular segments were packed with as many as 6 of these germ-like structures. Quantitation of dorsal paw skin from control and ΔN87βcat animals revealed an overall increase of 300% ± 30% in the density of invaginations relative to those generated by the hair follicles established during embryogenesis. While an average of two new invaginations arose between each pair of preexisting follicles at d24, this number is likely to be a conservative

estimate of the final outcome, because new invaginations continued to develop over time. Given that 1) subsequent hair cycles are not synchronized in the mouse, and 2) the morphology of the skin became grossly aberrant over time, the inventors limited the inventors' quantitations to the first hair cycle period.

The aberrant hair-germ like structures rapidly progressed to epithelioid cysts which were abundant in 3-4 month old skin. Projections stemming outward from these cysts were now clearly recognizable as hair follicles. These new follicles were extremely densely packed, often with no interfollicular epidermis between them. In contrast, an average of 20-30 interfollicular epidermal cells spaced each hair follicle in control skin of comparable body regions. Thus, while the total skin surface area was increased as a consequence of cyst formation, this increase was not nearly as great as the increase in follicle density that occurred.

Whether arising from dorsal paw skin or from backskin, the follicles arising *de novo* postnatally displayed characteristics that are normally only evident during embryonic follicle morphogenesis. The bulbar portions of the follicles encased mesenchymal condensates, morphologically analogous to dermal papillae, and many areas of these cysts were plentiful with sebaceous glands and hair shafts. Although less frequent, hair shaft formation was evident in some interfollicular hair germs of the dorsal paw skin as early as d24 postnatally.

Staining with antibodies against trichohyalin and hair-specific keratins confirmed that the differentiation occurring within many early stage *de novo* hair follicles was organized and hair-specific. Counter-staining with anti-K14 or anti-K5 antibodies revealed that interfollicular and upper ORS invaginations expressed these basal keratins, and that as *de novo* hair follicles differentiated, expression was restricted to the ORS. This said, expression of differentiation markers was not always organized in these structures. The epithelium surrounding the cysts stained positive for hair-specific markers, as did some of the morphologically aberrant hair germs. Overall, a strong correlation existed between the ability of these *de novo* follicles to

maintain proper morphology and their maintenance of a proper program of terminal differentiation.

The epitheliod cysts with radial projection of hair follicle-like structures in ΔN87βcat mice bore a striking resemblance to human trichofolliculomas, well-differentiated hair tumors each typified by an epithelioid cyst containing densely packed and misangled hair follicles, and small hairs that emanate from cysts at odd angles relative to the skin surface (Fitzpatrick *et al.*, 1993). Scanning electron microscopy confirmed that of those hairs in the ΔN87βcat mice that actually broke the skin surface, the angling was aberrant, and hairs were frequently irregularly positioned and of dramatically varied diameters. Human trichofolliculomas generally arise as isolated tumors; in contrast, development of trichofolliculoma-like tumors was rampant in the inventors' transgenic mice, eventually giving rise to deep skin folds over the entire body. The striking resemblance of ΔN87βcat skin to these human tumors leads to the postulate that an alteration of a gene involved in β-catenin regulation may be causative for trichofolliculoma tumors in humans.

EXAMPLE 6

De Novo Postnatal Hair Follicles in ΔN87βcat Mice Are Misangled and Have Apolarized *Sonic Hedgehog* Expression

Mammalian hairs are polarized at a specific angle relative to the skin surface (Hardy, 1992). A striking feature of the *de novo* hair follicles formed in ΔN87βcat skin was their inappropriate angling and loss of polarity. While the mechanisms controlling this process have not yet been elucidated, it seems likely that follicles sense position by some factor(s) that is itself polarized. *Sonic hedgehog* is a factor which is expressed in the hair bulb, but only in a patch at the side of the matrix which is closest to the skin surface (Bitgood and McMahon, 1995). When coupled with the knowledge that the *Wnt* pathway is often associated with the *sonic* hedgehog (*Shh)* pathway (Willert and Nusse, 1998), the inventors wondered whether a) *Shh* might be induced in the ΔN87βcat-generated hair germs, and b) whether misexpression of ΔN87βcat might have resulted in a change in *Shh* polarization. *Shh* mRNAs were

indeed induced early in the formation of aberrant ΔN87βcat hair germs in interfollicular dorsal paw skin. As in wild-type follicles, *Shh* mRNAs were restricted to the developing matrix cells in contact with the dermal papilla; however, in contrast to normal hair follicles, the patterns of *Shh* mRNA expression were most peculiar. In some hair germs, such as the one shown in frame B, expression appeared to be random, with some matrix cells expressing at significantly higher levels than others. In other cases, such as the one shown in the inset to frame B, expression occurred in discrete patches on both sides of the matrix.

Activated *Shh* binds to *patched*, a membrane-bound receptor which indirectly represses its own gene transcription in the absence of *Shh* (Hooper and Scott, 1989; Ingham *et al.*, 1991). Thus, upregulation of *Ptc* mRNA expression is a biochemical indicator of *Shh* activation (for review, see Ingham, 1993; Oro *et al.*, 1997). Whether interfollicular or ORS-derived, the matrix cells of developing *de novo* hair germs displayed marked hybridization to a didegoxygenin-labeled *Ptc* cRNA probe.

Early in the formation of these follicle-like structures, *Shh* and *Ptc* mRNAs were restricted to the matrix cells at the follicle base. However, as the follicles became progressively more irregular and trichofolliculomas began to develop, *Shh* and *Ptc* mRNA expression also became progressively more aberrant. Expression was still seen in matrix cells, but some irregular follicles displayed additional expression along the length of their outer root sheaths. This expanded pattern of *Shh* and *Ptc* expression arose in cells that were also positive for the basal keratins, and thus for K14 promoter activity and ΔN87βcat expression. Like the formation of the hair germs themselves, expression required some additional, as yet unidentified factor, since not all cells positive for transgene expression displayed *Shh* and *Ptc* induction.

Prior to the start of the first postnatal hair cycle, most if not all embryologically established hair follicles maintained polarized *Shh* expression in a fashion indistinguishable from wild-type. Even after hairs began to cycle and *de novo* follicles appeared, preestablished follicles could often be distinguished in that they maintained their orientation and polarization. Notably, these follicles also maintained

proper polarization of *Shh*. Thus, within the skin of ΔN87βcat mice, particularly within the early stages after initiation of the first postnatal hair follicle, a mixture of follicles were seen: some with wild-type orientation and proper *Shh* polarization, and some with random angling and apolarized *Shh* expression. As the phenotype progressed and the number of *de novo* hair follicles increased, the percentage of properly oriented follicles with proper *Shh* polarization seemed to decrease. Taken together, these findings suggested that *Shh* polarization might be a key event in follicle angling within normal skin.

EXAMPLE 7

Older ΔN87βcat Mice Develop Pilomatricomas

As ΔN87βcat mice aged, they developed visible, large tumors (>1 cm dia.), which by histology were much less differentiated in appearance than trichofolliculomas. A well circumscribed ring of highly mitotic, darkly staining basophilic cells with scant cytoplasm marked the borders of the tumors. These cells resembled hair matrix cells and were clearly distinct from basal epidermal and ORS cells. Their identity was confirmed by their expression of several markers of hair matrix cells, including *Lef-1, Shh* and *patched* (FIG. 8E and FIG. 8F; shown are the data for *Ptc*). *Ptc* levels superseded those of surrounding follicles, a feature also seen in human basal cell carcinomas (Oro *et al.*, 1997). However, in contrast to BCC, the centers of these less differentiated ΔN87βcat tumors were *Ptc* negative. In addition, the centers were filled with anucleated cellular ghosts, which bore a striking resemblance to "shadow" cells, the hallmarks of pilomatricomas, common hair matrix tumors in humans (Fitzpatrick *et al.*, 1993).

Shadow cells are normally a constituent of the hair medulla and are rarely seen in other skin tumors. Pilomatricomas occurred in a number of the older ΔN87βcat mice, but are extremely rare in normal mouse colonies. These data imply that pilomatricomas are derived from trichofolliculomas and are dependent upon ΔN87βcat expression, but that an additional gene mutation is required for their formation.

EXAMPLE 8

Clinical Trials for the Use of Agents Providing β-Catenin Activity in the Treatment of Alopecia

This example describes a protocol to facilitate the treatment of patients suffering from alopecia using an agent providing β-catenin activity alone or in combination with other hair growth-promoting agents.

A composition of the present invention is typically administered topically or parenterally by intradermal or subcutaneous injection. The agent providing β-catenin activity may be delivered to the patient before, after, or concurrently with other hair growth-promoting agents.

A typical treatment may comprise a single administration or a series of administrations of an agent providing β-catenin activity, either daily for several days, weekly for several weeks, or monthly for several months.

The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing β-catenin clinical trials.

Patient groups may consist of men with good general health and suffering from androgenetic alopecia (male pattern baldness) or alopecia areata who have not used a hair growth-promoting agent such as minoxidil in the past 6 months. Hair density can be measured by counting the number of terminal hair shafts per unit area or by evaluation of photographs (Olsen and DeLong, 1990; Gilhar *et al.*, 1990). Other methods of measuring hair growth such as use of optical microscopy and image analysis or by measuring the anagen ratio and hair diameters have been published (Tsuji *et al.*, 1994; Hayashi *et al.*, 1991). Hair measurements will be taken prior to administration of the agent providing β-activity and at monthly intervals for a period of several months.

EXAMPLE 9

Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation

In the present example, the relative patterns of Lef1 and TCF3 expression and localization during hair follicle development and in the postnatal hair cycle is elucidated. In addition, by engineering mice expressing a transgene whose expression is dependent upon activated Lef/TCF and β-catenin, the inventors identifies which cells receive and respond to a Wnt or equivalent signal at specific times within the developing and postnatal hair follicle. Transgene promoter activity in mice that are otherwise genetically wild type and in mice that express a constitutively active form of β-catenin was examined. The results reveals new insights into the regulation of cell fate commitments during hair follicle morphogenesis and differentiation. Transient Lef/TCF target gene activation was directly implicated in the initiation step of hair follicle morphogenesis in embryonic development, and also in the conversion of a subset of proliferating matrix cells to postmitotic hair-shaft-forming cells. Finally, evidence is provided that β-catenin stabilization can result in the activation of TCF3-positive cells within the putative stem cell compartment of the hair follicle. These studies further confirm the role of Lef/TCF and β-catenin in hair morphogenisis and possibilities of the invention.

MATERIALS AND METHODS

Generation of transgenic reporter mice

Plasmid pTOPGAL was engineered by replacing the luciferase reporter gene of pTOPFLASH (Korinek *et al.*, 1998a) with the bacterial *lacZ* gene. The TOP promoter consists of three multimerized Lef/TCF consensus binding sites and the c-fos minimal promoter (*Xba*I fragment) with the promoter-less pNASS(m) (*lacZ*) plasmid (linearized with *Spe*I). Plasmid pK14-ΔN87βcat was engineered as described herein. Transgenic mice harboring K14-ΔN87βcat and TOPGAL or TOPGAL alone were made as previously described (Vassar *et al.*, 1989), and transgene integration was verified using PCR™ analysis of tail DNAs. Transgene expression was verified

by X-Gal staining of tail samples β-galactosidase; Byrne *et al.*, 1994) and by phenotype ΔN87βcat.

X-Gal assays on frozen tissue sections

Frozen sections (10 μm) were fixed with 0.2% glutaraldehyde for 2 min. After washing 7-8 times in 1× PBS, slides were then transferred into Tissue Stain Base solution (Specialty Media) with X-Gal substrate at a final concentration of 2 mg/ml. Staining was performed in the dark at 37°C for 6-8 hr and, after mounting in 80% glycerol, samples were visualized by Nomarski optics with a Zeiss axiophot microscope. After photographing slides, coverslips were removed and slides were treated sequentially with Hematoxylin for 2 min, water rinse, eosin for 5 min and a final water rinse. Slides were then remounted and tissue sections were photographed as before.

Immunohistochemistry

Frozen sections of tissues were subjected to indirect immunofluorescence as described herein. For detection of TCF3, antigen unmasking was performed by autoclaving 4% paraformaldehyde-fixed tissue sections in 10 mM sodium citrate, for 2 min. Primary antibodies used were rabbit anti-Lef1 (van Genderen *et al.*, 1994); mouse monoclonal anti-TCF3 (Barker *et al.*, 1999); mouse monoclonal anti-trichohyalin (AE15; Manabe *et al.*, 1996) and anti-hair keratin (AE13; Lynch *et al.*, 1986); guinea pig anti-K5 (Byrne *et al.*, 1994); rabbit anti-Ki67 (proliferation marker). Fluorescence-conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA). Slides were mounted with antifade and visualized by Zeiss confocal microscopy.

In situ hybridization

Digoxigenin-labeled cRNAs were synthesized according to the manufacturers' instructions (Boeringer Mannheim Biochemicals, Indianapolis, IN). A 517 bp mouse *Lef1* cDNA, lacking the conserved DNA-binding domain, was subcloned as an *Eco*RI-*Bam*HI fragment into pCRII, and the cRNAs were made by either *Eco*RV linearization and SP6 RNA polymerase transcription, or *Bam*HI linearization and T7

RNA polymerase transcription. An analogous vector was made using the unique sequences of the mouse *Tcf3* cDNA (Korinek *et al.*, 1998a).

RESULTS

Generation of TOPGAL and K14-ΔN87βcat double transgenic mice

A transgenic mice expressing ΔN87βcat, a constitutively activated form of β-catenin, under the control of the keratin 14 (K14) promoter which is upregulated at embryonic day 14.5 (E14.5) in the basal layer of epidermis, the ORS and the bulge has been generated. At the initiation of the first postnatal hair cycle of these mice, the skin produces interfollicular epithelial invaginations, which sometimes resemble primordial hair germs and develop into bona fide hair follicles.

To first assess whether this striking action of ΔN87βcat *in vivo* is mediated through the activation Lef/TCF-responsive target genes, a second transgene was engineered, this one driving *lacZ* under the control of a promoter that contains multimerized Lef/TCF-binding motifs upstream from a minimal promoter (FIG. 4A). The transgene, referred to here as TOPGAL, is a derivative of TOPFLASH, whose expression is dependent upon stabilized β-catenin and is comparably responsive to TCF3 and Lef1 (Korinek *et al.*, 1998a). In cultured keratinocytes, TOPGAL, but not a version with mutated Lef/TCF binding sites (FOPGAL), behaved similarly and was superactivated when co-transfected with vectors expressing ΔN87βcat and hLef1, but not with either alone (FIG. 4B). With this verification, engineered mice transgenic for both K14-ΔN87βcat and TOPGAL were made. Two independently derived lines were generated and were bred to the F1 stage to avoid the possibility of mosaicism. Both lines behaved similarly in all assays performed.

Transgenic mouse skin was analyzed for β-galactosidase activity at 28 days of age, when many hair follicles were in anagen, and when there was a prevalence of interfollicular epithelial invaginations caused by ΔN87βcat expression. TOPGAL expression in skin from transgenic mice which were also positive for K14-ΔN87βcat.

The K14-þN8• þcat transgene and its behavior in mice has been described herein. Sections (10 þm) of the toe skin from 28-day-old double transgenic mice were assayed for þ-galactosidase activity using the X-gal assay. At all ages, control skins were negative for blue stain. Epidermal basal cells were detected to have activated the TOPGAL transgene; as well as a de novo hair germ (hg), induced artifactually by þN8• þcat expression, along with an epidermal cell that has activated the TOPGAL transgene. þN8• þcat-induced epithelial invaginations expressing TOPGAL were detected. As judged by X-gal staining, β-galactosidase activity was detected in a number of epithelial cells in the skin of these transgenic mice.

No blue cells were seen in skin from either wild-type or K14-ΔN87βcat transgenic mice, indicating that blue staining was a faithful measure of TOPGAL reporter activity in these mice.

Approximately 5-10% of the basal epidermal cells of 28 day K14-ΔN87βcat/TOPGAL transgenic mouse skin scored positive for β-galactosidase activity. In addition, postnatal de novo hair germs, unique to ΔN87βcat transgenic skin, stained blue, as did the flower-like epithelial invaginations that often followed the initial signs of ΔN87βcat-induced hair germ formation. TOPGAL activities in epidermis and in postnatal interfollicular epidermal invaginations were not seen when mice expressed only TOPGAL and not ΔN87βcat. These results provide strong evidence that transactivation of Lef/TCF- dependent target genes is induced in the epithelial invaginations and de novo hair follicles that arise as a consequence of K14-ΔN87βcat expression. Additionally, the study suggests that an endogenous Lef/TCF family member combined with ΔN87βcat to activate TOPGAL in these invaginations.

Only a subset of K14-ΔN87βcat-expressing basal cells stained blue. This suggests a heterogeneity in the potential for basal cells within the epidermis to transactivate Lef/TCF-regulated promoters. This heterogeneity could stem from differences in endogenous Lef/TCF levels and/or their phosphorylation states

(Ishitani *et al.*, 1999), or differences in the levels of inhibitory factors such as the GROUCHO/TLE, CBP and C-terminal binding protein (CtBP), all of which interact and interfere with the action of TCF/Lef family members (Bienz, 1998; Brannon *et al.*, 1999). These possibilities are explored in some of the studies presented below.

TOPGAL is active in a subset of *Lef1*-expressing embryonic skin cells at the start of hair follicle morphogenesis

Lef1 mRNAs are expressed early in embryonic mouse skin in both the ectodermal placodes and in the underlying dermal condensates (Zhou *et al.*, 1995), and it was reported that of the Tcf family members, TCF3 is also expressed in hair follicles (Barker *et al.*, 1999). *Lef1* and *Tcf3* behave very similarly in TOP promoter assays in at least some cells *in vitro*, and both are activated by the presence of stabilized β-catenin (Korinek *et al.*, 1998a). TOPGAL activation was used as an in vivo assay to understand how Lef1 and TCF3, presumably in conjunction with endogenously activated β-catenin, might be involved in normal hair follicle morphogenesis during embryonic skin development. To this end, mice were engineered harboring only the TOPGAL and not the K14-ΔN87βcat transgene. The TOPGAL transgenic mice were used for the remaining studies reported herein this example. Two lines were generated, and they behaved similarly in all assays.

The patterns of Lef1 protein and β-galactosidase activity assays in embryonic skins of F1 transgenic mice and correlated this with TOPGAL promoter activity were examined. Timed pregnancies of F1 mice harboring only the TOPGAL transgene were made to compare Lef1 protein with TOPGAL promoter activity during hair follicle development. Transgenic skin sections depicting the pregerm (PG) stage, where the follicles are merely ectodermal placodes and underlying dermal condensates, the hair germ/plug stage (hg), where epithelial downgrowth has been initiated and the early developing hair follicle stage (hf), where a pocket of dermal papilla cells have been encased by matrix cells to form a bulb-like structure were examined. Skin of a *Lef1* knockout newborn was used as a control for the antibody stainings. Sections were assayed for anti-K5 (K5) and/or anti-Lef1 (Lef1)

immunofluorescence or þ-galactosidase activity. Sites of blue ectodermal and mesenchymal cells corresponded roughly in frequency and distribution to Lef1-intense sites, and the absence of blue staining in developing hair germ/plug were noted.

Indirect immunofluorescence revealed anti-Lef1 antibody staining throughout the basal layer and developing pregerms (placodes) of embryonic skin epithelium. Staining was consistently more intense in the pregerms than in other areas of the ectoderm. Staining was also seen in the underlying dermal condensates. The distinction between epithelium and mesenchyme was best visualized by co-staining tissue sections with anti-K5, specific for the cytoplasmic keratin network of the epithelial cells. Moreover, from this double labeling, it was clear that the majority of anti-Lef1 staining was nuclear, at least in the epithelial component of the pregerm. Finally, no anti-Lef1 staining was seen in control skin from the *Lef1* knockout mouse (van Genderen *et al.*, 1994), verifying the specificity of the antibody. This was also confirmed by immunoblot analysis (Kratchowil *et al.*, 1996). Overall, these studies were consistent with the *in situ* hybridizations showing *Lef1* mRNA expression in the pluripotent ectoderm, and in both epithelium and mesenchyme of early hair germs (Zhou *et al.*, 1995). Interestingly, as judged by β-galactosidase activity, TOPGAL activation occurred in both epithelium and dermal condensates of hair pregerms. Blue-stained cells were quite evenly distributed at these two sites in the embryonic basal layer, and correlated well with the frequency and location of sites where anti-Lef1 antibody staining was pronounced. These studies indicated that Lef/TCF was able to act as a transcriptional activator at a time when both epithelium and mesenchyme were being programmed to form a hair follicle. Interestingly, within a pregerm, TOPGAL expression was often more prominent in either ectoderm or mesenchyme, raising the possibility that activation in one cell might promote activation of the other. This notion was attractive, given the documented series of mesenchymal and epithelial cross-signaling that occurs at these early stages of follicle commitment.

While β-galactosidase activity seemed to be restricted to a subset of pluripotent embryonic basal cells, Lef1 was readily detected in the nuclei of most if

not all cells within the basal layer. Moreover, nuclear Lef1 could be seen in these basal cells throughout embryonic development, *i.e.*, encompassing the time when ectoderm was pluripotent and able to choose between epidermal and follicle cell fates. This finding indicated that either the threshold levels of Lef/TCF factors were not sufficient to activate TOPGAL in many of these cells, or that an additional signal, perhaps a Wnt, was necessary to stabilize β-catenin and enable downstream target genes to be expressed.

During the next phase of follicle development, as the cells proliferated to form the hair germs (more advanced germs are sometimes referred to as plugs or pegs), TOPGAL expression was no longer detected. Anti-Lef1 staining was weak or absent not only in the stalk of the growing hair germ, but also in the basal epidermal cells adjacent to it. Instead, Lef1 seemed to be concentrated at the leading edge of the growing follicle, where it was detected both the developing matrix cells and dermal papilla. This was visualized by double immunofluorescence labeling with anti-K5, restricted to the epithelium. K5 expression is downregulated at the leading edge of the hair germ and in the matrix cells of the follicle (Kopan *et al.*, 1989), this distinction was still more difficult than it was at the pregerm stage. Overall, the studies agreed with the prior *in situ* hybridization studies, reporting expression of *Lef1* mRNAs in both epithelium and mesenchyme at the leading edge of developing follicles (Zhou *et al.*, 1995). Additional evidence to further confirm mesenchymal and epithelial expression of *Lef1* is described herein.

Concomitant with the completion of follicle morphogenesis, a marked decline in anti-Lef1 antibody staining in postnatal basal epidermal cells was noted, a finding consistent with *Lef1* mRNA expression patterns (Zhou *et al.*, 1995). Thus, a correlation seemed to exist between loss of pluripotency and a loss of *Lef1* expression in the basal layer. The downregulation of *Lef1* in postnatal epidermis provided further evidence that developmentally regulated TOPGAL expression is associated with events relating to hair follicle morphogenesis and not epidermal differentiation.

***Lef1* mRNAs are primarily expressed in the follicle bulb, while TOPGAL is induced in the differentiating hair shaft precursor cells**

Expression of *Lef1* mRNAs and TOPGAL in developing follicles that have formed the precursor cells of the hair shaft was examined. Skin sections were obtained from F1 generation TOPGAL transgenic mice at various developmental ages (E16.5 to postnatal day 9). Sections were either hybridized with digoxigenin-labeled antisense *Lef1* cRNAs or processed for þ-galactosidase activity assays. E16.5 skin was from ventral torso and from dorsal torso, and no differences in *Lef1* mRNA or TOPGAL expression were observed in whisker and body hair follicles at equivalent stages of morphogenesis.

TOPGAL expression reappeared in transgenic follicles beginning at E16.5, and was dramatically upregulated in both whisker and body hair follicles after birth. By this time, *Lef1* mRNA expression was beginning to weaken in the basal layer of epidermis, but increased in the hair bulbs, where it concentrated in the matrix and precortex for earlier embryonic *Lef1* mRNA expression data (Zhou *et al.*, 1995). Appreciable β-galactosidase activity was detected only in a subset of these *Lef1* mRNA-expressing cells. This triangular-shaped pocket of blue-stained cells appeared to be the precortex, *i.e.*, the precursor cells to the hair shaft. In postnatal skin, TOPGAL expression was strongly upregulated in the postnatal anagen follicles. The difference between *Lef1* mRNA localization and TOPGAL expression became even more pronounced by 6-9 days, when the well-established zone of transcriptional activity in the follicle broadened to encompass the upwardly migrating, differentiating cells (Kopan *et al.*, 1989; Zhou *et al.*, 1995; Dunn *et al.*, 1998). At these times, the majority of skin *Lef1* mRNAs concentrated in the follicle bulbs, although two lines (*e.g.*, a concentric ring in the three-dimensional follicle) of *Lef1* mRNA-expressing cells were seen extending from the bulb upward. In contrast, the strongest TOPGAL expression was detected in the differentiating cells above each hair bulb. This said, the two lines of cells expressing *Lef1* mRNAs also expressed TOPGAL, creating a narrow zone of overlap between the two patterns.

Biochemical evidence to establish the identity of the differentiating cells that express both TOPGAL and *Lef1* mRNAs was later obtained. However, at this point, it was thought that the differentiating cells at least included the precursor cells of the hair shaft, since clipped hairs of postnatal TOPGAL transgenic animals stained faintly blue when subjected to β-galactosidase activity assays. This blue staining was not seen in hairs from control mice that lacked the transgene, indicating that in the TOPGAL mice, it must have stemmed from residual β-galactosidase activity produced at a time when the differentiating cells of the developing hair shafts were still metabolically active. The Lef1-binding motifs previously noted in the hair-keratin-specific promoters (Zhou *et al.*, 1995) are likely to be functionally relevant to the expression of the hair keratin genes in the precortex, cortex and cuticle. The expanding pattern of β-galactosidase activity seen in the 9 day postnatal follicle was similar to the pattern of hair-specific keratin mRNA expression in anagen-stage rodent follicles (Kopan *et al.*, 1989; Zhou *et al.*, 1995).

Lef1 mRNA expression persisted throughout anagen, but then declined dramatically at catagen and telogen, as hair growth ceased and follicles regressed. β-galactosidase activity persisted in the hair shaft cells into telogen but, by 18 days postnatally, only faint staining was detected. Similar patterns of blue staining were seen in postnatal follicles at different body sites, including body, tail and whisker follicles. This was surprising, given that whiskers are completely lacking in *Lef1* knockout mice, whereas body hairs were only partially compromised (van Genderen *et al.*, 1994). This difference might stem from variations in the importance of Lef/TCF activation in the waves of embryonic follicle development that give rise to different hair types (Headon and Overbeek, 1999).

LEF1/TCF3 protein in the follicle

Given the notable difference between *Lef1* mRNA and TOPGAL expression in the postnatal hair follicle, studies were designed to evaluate whether TCF3 protein, known to be expressed in skin (Barker *et al.*, 1999), might be present in the precortex and account for the activation of TOPGAL in these cells. To address this question, sections of newborn TOPGAL transgenic mouse skin were subjected to

β-galactosidase activity assays, and anti-TCF3 immunofluorescence. Skins were taken from TOPGAL transgenic mice at t=0 (tail skin), or 9 days (dorsal torso) and processed for TOPGAL expression (X-gal; counterstained with Hematoxylin and Eosin) or indirect immunofluorescence. Antibodies used were: TCF3 (green), Lef1 (green), K5 (red), Ki67 (green), Dapi (blue). K5 is a marker of the outer root sheath (ORS); Ki67 is a nuclear marker of proliferating skin cells. Brightly stained Lef1 flat nuclei were visualized, more diffuse Lef1 staining of matrix and also the immunofluorescence negative zone that separates the K5-positive ORS cells from the Lef1-stained cells was noted. This zone is likely the inner root sheath (IRS), which can be distinguished morphologically from the precortex.

TOPGAL expression occurs as matrix cells differentiate, concomitant with an increase in intensity of nuclear anti-Lef1 immunofluorescence and in the apparent absence of TCF3. In contrast to TOPGAL, which was expressed in the precortex of newborn tailskin follicles, TCF3 was confined to the outer root sheath. Double immunofluorescence staining with antibodies against the cytoplasmic keratin K5 (red) revealed that the anti-TCF3 staining (green) was clearly nuclear. Thus, despite the fact that TCF3 and Lef1 behave similarly with stabilized β-catenin in TOP-promoter assays in lymphocytes in vitro (Korinek *et al.*, 1998a), TCF3 did not seem to coincide with TOPGAL expression in skin *in vivo*. The inactivity of TCF3 may be a reflection of its interaction with the CtBP repressor protein, known to act selectively on TCF3 and not Lef1 (Brannon *et al.*, 1999).

Whether the pattern of Lef1 might help explain the apparent differential expression of *Lef1* mRNAs and TOPGAL expression was examined next. Serial sections of the follicle were stained with anti-Lef1 antibodies. Close inspection revealed a surprising staining pattern of anti-Lef1 that differed considerably from the anti-Lef1 pattern seen in the embryonic follicle as well as the pattern seen by *Lef1* cRNA *in situ* hybridization. In the dermal papilla, the intensity of anti-Lef1 staining seemed weaker than had been observed in embryonic dermal condensates. Additionally, in the matrix cells of newborn follicles, where *Lef1* mRNAs were most strongly expressed, anti-Lef1 staining was weak and somewhat diffuse. However, in

the precortex, anti-Lef1 staining was intense and more sharply nuclear. Thus, whereas *Lef1* mRNAs were more abundant in the undifferentiated matrix cells, Lef1 appeared to accumulate in the differentiating precortex cells. Moreover, whereas TOPGAL and *Lef1* mRNA expression patterns only overlapped in a narrow zone, TOPGAL expression correlated well with the pattern of intense nuclear anti-Lef1 staining. Taken together, it seems likely that some factor, possibly stabilized β-catenin, is responsible for the apparent accumulation of Lef1 and activation of TOPGAL in the precortex.

Staining of a serial section of these follicles with antibodies against the proliferation-associated antigen Ki67 (Smith *et al.*, 1995) revealed that the proliferating matrix cells were those that stained more faintly for anti- Lef1, whereas the precortex cells, which stained very strongly for anti-Lef1, did not stain with anti-Ki67. These data are consistent with prior [^{3}H] thymidine labeling (Wilson *et al.*, 1994) and histone H3 *in situ* hybridizations. The studies provide compelling evidence that the site of TOPGAL expression and intense nuclear Lef1 antibody staining is in the non-proliferative compartment at the follicle base. This finding was unexpected given reports that TCF/Lef and β-catenin transcription complexes can activate *c-myc* and *cyclin D* genes (He *et al.*, 1998; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999), and the well-established role of activating β-catenin mutations and cancer (Clevers and van de Wetering, 1997; Chan *et al.*, 1999.

The distinction between diffuse anti-Lef1 staining in the matrix and more focused nuclear staining in the precortex became even more prominent at 6 and 9 days. During this time, nuclear Lef1 expanded to include one to two layers of cells above the bulb. Co-staining with anti-K5 revealed a zone of immunofluorescence negative cells between the K5-positive ORS cells and the Lef1-positive layers. This negative zone adjacent to the ORS was likely the inner root sheath (IRS), indicating that the one to two layers of cells with flat nuclei were likely to be the differentiating cortical and/or cutical layers of the hair shaft, previously found to express TOPGAL. Additional evidence in support of this identification is provided below.

Biochemical identification of the TOPGAL-expressing cells

To verify that the TOPGAL/strongly nuclear Lef1-positive cells in the mature follicle were also the ones that expressed the hair specific keratins, well-characterized monoclonal antibodies that are highly specific for either the hair shaft keratins (AE13) or the IRS marker trichohyalin (AE15) were used (Manabe *et al.*, 1996; Lynch *et al.*, 1986). Backskins from TOPGAL transgenic mice were taken at various ages (nb, day 6 and day 9), and tissues were processed for either þ-galactosidase activity (X-gal, and/or counterstained with hematoxylin and eosin), or indirect immunofluorescence. Antibodies used were: Lef1 (green), AE13 against hair-specific keratins (purple), K5 specific for the ORS of the follicle (red), AE15 specific for the IRS (purple). A filter was applied across the images of immunofluorescence. This distorts the actual coloring slightly, but allows the reader better visualization of the blue secondary antibody as purple and the overlap of blue AE13 and green Lef1 labeling as turquoise (more Lef than AE13) and white (more AE13 than Lef1). Sections through the heart of the follicles and through the heart of the follicle bulbs, but at a slight angle grazing through the IRS rather than the hair shaft were examined.

Biochemical identification of the strongly Lef1-positive, TOPGAL-expressing cells as those that begin to express the hair-specific keratins and not inner root sheath markers. Serial sections stained for β-galactosidase activity, intense nuclear anti-Lef1 staining and anti-hair keratin (AE13) staining were clearly overlapping in the precortex region of newborn mouse skin. In contrast, another serial section of the same newborn follicle stained with anti-trichohyalin (AE15) and anti-Lef1 revealed that these patterns were mutually exclusive. The overlapping patterns of β-galactosidase activity, anti-Lef1 and AE13, but not AE15, were also seen later in anagen by the 6 and 9 day postnatal skin samples. Again, serial sections were taken so that the same follicle could be examined following various treatments. These studies unequivocally identify the TOPGAL-expressing cells as precortex and not IRS, and further demonstrate that hair keratin expression initiates in the cells that express TOPGAL.

TOPGAL activation at the initiation of the new hair cycle

The hair follicle is derived from a series of mesenchymal and epithelial cues transmitted in early embryonic development (Hardy, 1992). These signals have been predicted on the basis of tissue recombination experiments conducted by early developmental biologists (Sengel, 1976; Hardy, 1992). The postnatal hair cycles through periods of growth (anagen), rest (catagen) and regression (telogen). The transition from telogen to the new cycle is thought to depend upon a stimulus from the dermal papilla to the bulge, a permanent compartment of epithelial stem cells surrounding the dead hair club from the prior cycle. This results in epithelial proliferation to form the secondary hair germ (2° hg).

Contiguous with the ORS and localized at the transition zone between permanent and cycling portions of the hair follicle is the bulge, the putative compartment of follicle stem cells (Lavker *et al.*, 1991). This compartment of epithelial cells is not needed for establishing the embryonic hair follicle, but it is required for the initiation of each new hair cycle, as stem cells are stimulated by an as yet unidentified signal, most likely from the dermal papilla (Lavker *et al.*, 1991). Wnt signaling and TCF/Lef transcription factors may be utilized at this stage of the hair cycle.

Activation of TOPGAL and expression of Lef1 and TCF3 at the start of the new hair cycle was examined. Tail skin or the haired portion of hindfoot skin from 28 days TOPGAL/K14-þN8• þcat mice, 28 days TOPGAL mice or a newborn TOPGAL mouse were sectioned and processed for either þ-galactosidase activity (X-gal in blue) or indirect immunofluorescence with anti-TCF3 (green), anti-Lef1 (green) anti-Ki67 (green) or anti-K5 (red). While no major differences in the two skin regions of 28-day mice were detected with regards to the biochemical assays employed here, some regional differences in the timing of the second hair cycle were observed. Thus, some follicles showed no outgrowth, while another follicle had produced a secondary hair germ (2° hg), and another follicle had progressed further into anagen, already producing a precortex. Several cells in the bulge region surrounding the old hair club are stained blue. Blue staining of the old hair club may reflect residual galactosidase

activity persisting in the dead hair cells; this section was stained overnight to verify the lack of staining in the 2° hg.

By 26-28 days, virtually all follicles in CD1 mice have entered their first postnatal hair cycle. Although follicles were also examined at earlier ages, the study concentrated on this age to optimize the likelihood that a follicle would be activated rather than dormant, a distinction that is difficult to make morphologically when follicles are at telogen or the transition stage from telogen to anagen. Despite the quite prominent synchrony of the first postnatal hair cycle, there are some differences in the timing of initiation due to such factors as regional variations (*e.g.*, whisker, torso, tail, limb skin), variations in the cycles of coat hair types, anterior-to-posterior waves of hair cycle initiation, and also the sex and birth time (± 0.5 day) of the animals (Kobori and Montagna, 1975; Wilson *et al.*, 1994; Headon and Overbeek, 1999).

In those 28-day follicles that did not yet show signs of the secondary hair germ, a few cells within the bulge sometimes stained blue for TOPGAL expression. The ability to detect a signal in this region was facilitated in mice transgenic for both TOPGAL and ΔN87β catenin. Only a few cells in each bulge area that stained blue was observed. This result was similar to that obtained for the initiation stage of follicle morphogenesis in embryonic skin.

The bulge region was examined for the presence of TCF3 and Lef1. Anti-TCF3 staining was seen within the bulge, where it was localized in cell nuclei. Nuclear TCF3 persisted at this location throughout all stages of the hair cycle, even in telogen, when the ORS below the bulge was absent. In the upper portion of the follicle above the bulge, and in epidermis, anti-TCF3 labeling was dramatically diminished.

At the initiation of the next hair cycle, anti-TCF3 was detected in the K5-positive cells that grew downward to form the secondary hair germ. However, at the leading edge of the germ, cells were weak or negative for anti-TCF3 staining. In

contrast to anti-TCF3, anti-Lef1 did not stain the bulge appreciably at any stage of the hair cycle. Just after the initiation of the next hair cycle, however, anti-Lef1 stained a subset of cells emanating down from the bulge to form the secondary hair germ. These cells were clearly positive for K5, distinguishing them from the dermal papilla, which remained anti-Lef1 positive throughout the hair cycle. In this regard, and in their intense nuclear staining, these developing matrix cells of the secondary hair germ resembled those of primary hair germs formed during embryonic development. A serial section from this follicle was stained with proliferation antigen anti-Ki67, revealing that the outer cell layer of the secondary hair germ was strongly positive. In contrast, the dermal papilla were negative for Ki67. A few Ki67-positive cells were occasionally seen above the secondary hair germ at the base of the old follicle. The proliferative capacity of these cells at the base has been described by Wilson *et al.* (1994), who showed that putative stem cells transiently incorporate labeled thymidine at this early anagen phase of the new cycle.

TOPGAL expression in this follicle was examined by staining another serial section of tissue. β-galactosidase activity was not detected in the growing hair germ. It was found at the base of the old hair club and, while this was never seen in non-transgenic skin, it was most likely due to residual enzymatic activity in the dead hair cells rather than new induction of TOPGAL. At a slightly later stage of anagen, as the secondary follicle began to form a bulb, β-galactosidase was detected in a triangular zone of cells, which appeared to be the developing precortex.

The pattern of anti-Lef1 staining at this stage of anagen was strikingly different from that at the slightly earlier stage of secondary hair germ formation. This was true not only for the epithelium but also for the dermal papilla. Thus, as the dermal papilla became engulfed by the follicle epithelium to make a bulb, the intensity of anti-Lef1 staining increased in the center where the precortex region developed, and decreased in both the developing matrix and the internalized dermal papilla. While these changes in the transition from embryonic to early postnatal development was observed, these changes were particularly striking when observed within individual samples of 28-day skin.

The changes in TOPGAL and anti-Lef1 staining were accompanied by changes in anti-K5 and anti-Ki67 patterns. During this transition, K5 became restricted to the ORS and was not expressed in matrix. Both ORS and matrix cells maintained marked proliferative activity, but clear zones of Ki67-negative cells were now seen in the follicle.

DISCUSSION

Activation of LEF1 as a transcription factor in both ectoderm and mesenchyme at the initiation stage of hair follicle morphogenesis and Wnt signaling

This example provides the strongest evidence to date that implicates Wnt signaling in mammalian hair development. This example defines two distinct roles for Lef/TCF activation of downstream target genes in initiating hair follicle morphogenesis. Early tissue recombination studies have indicated that the first signal in hair follicle development stems from the mesenchyme that instructs the ectoderm to form a placode (Sengel, 1976; Hardy, 1992). Based on the ability to detect TOPGAL in ectoderm, this first mesenchymal cue could either be a Wnt directly, or alternatively, a signal such as BMP-4, known to induce *Lef1* gene expression and influence hair organogenesis *in vitro* (Kratchowil *et al.*, 1996; Keranen *et al.*, 1998). While there may be additional Lef/TCF factors in this process, Lef1 but not TCF3 was detected at this early stage of development.

TOPGAL expression in the developing dermal condensate was observed and, since TOPGAL expression seemed to be predominantly in either ectoderm or dermal condensate, it seems that the two signals leading to Lef/TCF target gene expression are sequential, rather than simultaneous. One or more of the mesenchymal-ectodermal signals that lead to hair germ formation may be Wnts. Alternatively, the results indicate that the next dermal message, instructing committed epithelium to proliferate and form the hair follicle (Hardy, 1992), might not involve a Wnt, if indeed the TOP promoter provides a faithful and broad representation of Lef/TCF activation *in vivo*, as it is known to do in lymphocytes *in vitro*

(Korinek *et al.*, 1998a). Rather, based upon the block in the proliferation step of embryonic *Sonic hedgehog* null follicles (St. Jacques *et al.*, 1998; Oro *et al.*, 1997), *Shh* is a possible candidate for regulating this process.

Activation of the LEF1 transcription factor and hair shaft differentiation

Hair matrix cells are able to select morphologically and biochemically distinct differentiation pathways leading to the IRS and hair shaft (Hardy, 1992). The present studies provide among the first biochemical insights into how matrix cells select these programs of differentiation. Thus, while most if not all matrix cells express *Lef1* mRNAs and some Lef1 protein, only cells at the center of the bulb appear to accumulate Lef1 and activate downstream target genes concomitant with commitment to differentiate. The TOPGAL-expressing cells give rise to the hair shaft, while the surrounding cells choose an alternative pathway of differentiation and give rise to the IRS. The studies indicate that induction of *Lef1* expression is a characteristic of undifferentiated matrix cells, and that utilizing Lef1 to activate downstream target genes is a feature of matrix cells that become committed to a hair shaft differentiation program.

The finding that Lef1 activation plays a role in hair shaft differentiation provides an explanation for why so many hair-specific keratin genes possess Lef1-binding motifs in their upstream regulatory sequences (Zhou *et al.*, 1995). Testing the role of Lef1 in hair-specific keratin gene expression has been complicated by the lack of a suitable cell culture system for hair matrix cells, the cell type that gives rise to the hair shaft. *In vivo* studies in transgenic mice have revealed that mutation of the Lef1-binding site in one of these hair-specific promoters results in an apparent reduction in promoter activity (Dunn *et al.*, 1998). The finding that TOPGAL is expressed in precortical cells that give rise to the hair shaft provides compelling evidence that activated Lef1 is important in regulating genes that specifically give rise to the hair shaft.

Several Wnt mRNAs have been found in mature hair follicles. Of these, *Wnt-3* is of special interest in that its mRNAs are expressed in the precursor cells of the hair medulla (Millar *et al.*, 1999).

A role for TCF3 or LEF1 in activating follicle stem cells

A model to explain the hair cycle is that stem cells in the bulge become activated at the start of each new cycle, when they receive an as yet unidentified signal transmitted by dermal papilla cells that have retracted upwards at the end of the previous cycle (Hardy, 1992). The studies here have shown that nuclear TCF3 resides continually in the putative stem cell compartment within the bulge. In addition, although co-expression of ΔN87βcat was needed to detect strong TOPGAL activation in the bulge, a few cells within the bulge area of both TOPGAL and TOPGAL/ΔN87βcat follicles expressed β-galactosidase at or around the initiation of the first postnatal hair follicle. Lef1 also appeared to be activated at or soon thereafter, as judged by the fact that intense nuclear staining was concentrated in the secondary hair germ epithelial cells maintaining direct contact with the dermal papilla. This evidence indicates the possibility that TCF3/Lef1-mediated target genes may be expressed as an important step in stem cell activation. Additional studies using factors such as plucking to stimulate bulge cells (Wilson *et al.*, 1994), as well as measurements of TCF3's transactivation potential in ORS keratinocytes and/or bulge cells may be conducted.

Proliferation, tumorigenesis and activation of TCF/LEF1

The lack of appreciable TOPGAL expression in proliferating cells within the skin was surprising, given the well-documented presence of activating β-catenin mutations and APC mutations in a number of human cancers, including pilomatricomas, relevant to this study (Chan *et al.*, 1999). Indeed, the only known mammalian target genes presently known for activated β-catenin/Lef/TCF complexes are *cyclin D1* and *c-myc*, both protooncogenes important for cell proliferation (He *et al.*, 1998; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999). One possibility is that activation of proliferation-associated target genes by activated Lef/TCF factors may occur only when the natural mechanisms governing Lef/TCF/β-catenin regulation

are subverted, as in the case of β-catenin activating mutations. In this regard, the interfollicular downgrowths induced by the constitutively active β-catenin transgene, K14-ΔN87βcat, displayed strong TOPGAL activation in the double transgenic mice. These invaginating epithelial pockets grow very rapidly and ultimately lead to pilomatricoma tumorigenesis. When taken together with the finding that in normal postnatal follicles, activation of Lef/TCF transcription factor complexes seems to arise to change an epithelial cell's fate or differentiation state, it is contemplated that different threshold levels of Lef1/TCF activation may explain how TCF/Lef-mediated activation can induce a terminal differentiation program on the one hand, and tumorigenesis on the other.

EXAMPLE 10

β-catenin signaling can initiate feather bud development

Feather morphogenisis and hair morphogenesis are regulated by the same signalling pathways. The present example demonstrates that β-catenin and its signalling pathway are important in stimulating hair morphogenisis and the morphogenisis of other epidermal structures, such as feathers.

The role of β-catenin signaling in the skin of the experimentally accessible chicken embryo was examined. The change in the stability and subcellular localization of β-catenin can be monitored by immunohistochemical techniques to chronicle the activity of this pathway during development of a tissue (Schneider *et al.*, 1996). This signaling pathway was activated in a dynamic pattern throughout skin development from the onset of tract patterning through the generation of polarized buds. This pattern indicates roles for β-catenin signaling in placode formation, intra-bud patterning, and polarized outgrowth of the bud.

To evaluate these roles, the expression of a mutant form of β-catenin which serves to activate this pathway was forced using a retrovirus to generate patches of expression in developing skin (Funayama *et al.*, 1995; Capdevila *et al.*, 1998).

White Leghorn eggs (SPAFAS) were incubated at 37.63°C. Skin spanning the dorsal pterylae and femoral tracts was dissected in cold PBS and fixed overnight (4% paraformaldehyde, 4°C). After dehydration through graded sucrose and infiltration with OCT the skin was quick frozen, and alternating 8 μm sections were processed for immunohistochemistry or *in situ* RNA transcript detection. Endogenous β-catenin was detected with a monoclonal antibody (15b8, Sigma) diluted 1:500 in PBST followed by secondary detection with fluorescein-conjugated anti-mouse IgG. Sections were counterstained with 7-amino-actinomycin D or TOPRO 3 and images were captured on a Leica confocal microscope employing sequential scanning to eliminate bleedthrough between channels. *In situ* hybridization was as described by Morgan *et al.* (1998). Infected cells were detected with a polyclonal antisera to the viral p27 protein (SPAFAS), or with a monoclonal raised against the HA epitope tag (Babco).

The RCAS-β-catenin retrovirus was the generous gift of C. Tabin. Stocks of viral innoculum were generated as described by Morgan and Fekete (1996). A stock of 2×10^8 infectious units/ml was injected and embryos harvested as described by Noramly and Morgan (1998). The expression of viral transcripts alone was examined in a total of 31 embryos. Of these, 13 were harvested at day 6 of incubation, 9 at day 7, 6 at day 8 and 3 at day 9. Prior to day 8, no spatial restriction in infection was observed. However, preferential infection of the ectoderm of the feather bud primordia compared to interfollicular skin was observed after the dermal condensation has formed and the bud rudiment has begun to protrude from the ectoderm. Infection is also common in the apteric ectoderm at these stages. This pattern was observed with control RCAS viruses and apparently arises in part from transcriptional silencing of integrated viral genomes and increased resistance to reinfection as interfollicular ectoderm differentiates. Since this is a replication competent retrovirus, silencing is normally accompanied by rapid reinfection with virus produced by adjacent cells as the block to superinfection is lost.

A total of 16 day-12 and day-13 embryos were examined for gross phenotypic effects. To characterize the effect of exogenous activated β-catenin on endogenous

gene expression, whole-mount *in situ* analysis was performed on a total of 123 embryos. Of these 41 were examined for *BMP-2*, 28 for *Shh*, 15 for *BMP-4*, 19 for *Wnt7a*, 12 for *Lunatic fringe* and 8 for *TCF-1*. To correlate the effects on gene expression with the presence of stabilized β-catenin, these embryos were subsequently analysed for the presence of viral transcripts. To compare the extent of the expression of ectopic *Shh* with induced *BMP-2* expression, sequential detections of *BMP-2* and *Shh* were performed. 22 embryos were examined for for *BMP-2* transcripts using a digoxigenin-labeled probe and for *Shh* transcripts using a fluorescein-labeled probe; 22 embryos were examined with *Shh* digoxigenin-labeled probe first and then BMP-2 fluorescein-labeled probe. Control double detections of *BMP-2* (seven embryos) and Shh (two embryos) were also carried out. An additional 72 embryos were analyzed with feather bud markers not shown in this study and the results of these *in situ* hybridizations were consistent with those described in this invention. Probe templates were as described by Noramly *et al.* (1996); Song *et al.* (1996); Laufer *et al.* (1997); Morgan *et al.* (1998); Noramly and Morgan (1998). The TCF-1 probe was derived from nt 630-1400 of the chTCF-1 plasmid (Gastrop *et al.*, 1992). Six embryos were sectioned after whole-mount *in situ* hybridization as described by Noramly and Morgan (1998) to examine the morphology of the induced placodes. Representative samples were also sectioned to confirm the localization of induced gene expression to the ectoderm or dermis. Finally, virus was detected immunohistochemically on sections from an additional 8 embryos to confirm conclusions based on whole-mount analysis.

The construct was sufficient to induce bud formation since it does so both within presumptive feather tracts and in normally featherless regions where tract-specific signals are absent. It was also insensitive to the lateral inhibition that mediates the normal spacing of buds and can induce ectopic buds in interfollicular skin. However, additional patterning signals cooperate with this pathway to regulate gene expression within domains of stabilized β-catenin expression.

In contrast to the analogous study in the mouse, expression of this mutant protein in embryonic ectoderm is sufficient to induce the ectodermal gene expression

normally associated with early placode formation. These induced placodes can recruit underlying dermis to form feather buds. Furthermore, the asymmetric activation of this pathway during the normal progress of bud development is required for polarized outgrowth as ectopic activation within a bud can disrupt this process. These results indicate that activation of the β-catenin pathway initiates follicle development in embryonic skin and plays important roles in the subsequent morphogenesis of the bud. β-catenin signaling is contemplated as a positive activator of feather formation and endogenous Wnt signals may regulate its activity and localization to initiate dense dermis formation and placode development as well as to regulate polarized outgrowth of the bud.

* * *

All of the compositions, methods and apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions, methods and apparatus of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, methods and apparatus, and in the steps or in the sequence of steps of the methods described herein, without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method for inducing hair growth comprising providing β-catenin activity to a skin cell.

2. The method of claim 1, wherein the β-catenin activity is provided by providing a β-catenin polypeptide to the skin cell.

3. The method of claim 2, wherein the β-catenin polypeptide is ΔN87βcat.

4. The method of claim 3, wherein said β-catenin polypeptide is provided to the skin cell by introducing to the skin cell a polynucleotide encoding a β-catenin polypeptide.

5. The method of claim 4, wherein said polynucleotide is operably linked to a promoter.

6. The method of claim 5, wherein said promoter is selected from the group consisting of keratin promoters, involucrin promoters, filagrin promoters and loricrin promoters, CMV IE, SV40 IE, RSV, β-actin, tetracycline regulatable and ecdysone regulatable.

7. The method of claim 6, wherein said keratin promoter is selected from the group of K5 and K14.

8. The method of claim 4, wherein said polynucleotide is contained in a vector.

9. The method of claim 8, wherein said vector is a viral vector.

10. The method of claim 9, wherein said viral vector is selected from the group consisting of adenovirus, retrovirus, adeno-associated virus, vaccinia virus, and polyomavirus.

11. The method of claim 4, wherein said β-catenin polypeptide encoded by said polynucleotide is ΔN87βcat.

12. The method of claim 1, wherein the β-catenin activity is provided by providing to said skin cell a compound that increases β-catenin polypeptide levels in the skin cell.

13. The method of claim 1, wherein the β-catenin activity is provided by providing to said skin cell a β-catenin agonist.

14. The method of claim 1, wherein the β-catenin activity is provided by providing to the skin cell an agent that induces the expression of a β-catenin polypeptide encoded in said skin cell.

15. The method of claim 1, wherein the β-catenin activity is provided by providing to the skin cell an agent increases the stability of a β-catenin polypeptide in said skin cell.

16. The method of claim 1, wherein the β-catenin activity is provided by providing to the skin cell an agent that inhibits the degradation of β-catenin.

17. The method of claim 1, further comprising providing said skin cell with an agent that provides a factor that triggers the induction of hair growth by β-catenin

18. The method of claim 1, further comprising administration to skin of a method to initiate existing hair follicles to enter a synchronous hair cycle.

19. The method of claim 18, wherein said method of initiating a synchronous hair cycle is depilation.

20. The method of claim 18, wherein said method of initiating a synchronous hair cycle is administration of a chemical agent.

21. The method of claim 20, wherein said chemical agent is cyclosporin or FK506.

22. The method of claim 1, further comprising providing said skin cell with an agent that provides *Lef-1* activity to said skin cell.

23. The method of claim 1, wherein said cell is a keratinocyte.

24. The method of claim 1, wherein said cell is a human skin cell.

25. The method of claim 1, wherein the β-catenin activity is delivered to said skin cell *ex vivo* and then said skin cell is returned to the skin of the donor of said skin cell as an autograft.

26. The method of claim 1, wherein the β-catenin activity is provided in a pharmaceutically acceptable formulation.

27. The method of claim 1, wherein the β-catenin activity is provided to said skin cell by topical application in a pharmaceutically acceptable formulation.

28. The method of claim 1, wherein said topically applied pharmaceutically acceptable formulation contains liposomes.

29. A method for inducing *de novo* hair morphogensis comprising providing β-catenin activity to a skin cell.

30. A method for the treatment of alopecia comprising providing β-catenin activity to a skin cell.

31. A method for stimulating hair growth comprising providing β-catenin activity to a skin cell.

32. A method for the prevention of hair loss comprising providing β-catenin activity to a skin cell.

33. A method for estimating the propensity for developing alopecia, comprising evaluation of β-catenin expression in a skin cell.

34. A method for estimating the propensity for developing alopecia, wherein the responsiveness of a skin cell to β-catenin activity is evaluated.

35. A method for screening for hair growth disorders, wherein the level β-catenin expression in a skin cell is evaluated.

36. A method for screening for hair growth disorders, wherein the responsiveness of a skin cell to β-catenin activity is evaluated.

37. A method for screening agents that increase β-catenin levels in a skin cell, wherein the level of β-catenin expression is measured within said skin cell.

38. A method for screening agents that deliver β-catenin activty in a skin cell, wherein the translocation of Lef-1 to the nucleus within said skin cell is monitored.